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13. ABSTRACT *(Maximum 200 Words)*

Mutations in BRCA2 are responsible for about 35% of familial breast cancers and also a proportion of familial ovarian cancers. Both BRCA2 and BRCA1 proteins were shown to have transcriptional activation domains and also shown to be associated with RNA polymerase suggesting that these proteins may function as transcriptional factors and have a role in the regulation of transcription. Recent studies on enzymes responsible for histone acetylation and deacetylation revealed that some of the transcriptional factors function as histone acetyl transferases. Since BRCA2 showed transcriptional activation properties, we tested whether BRCA2 is associated with histone acetyltransferase. Our results suggested that BRCA2 is associated with histone acetyltransferase (HAT) activity. We propose to test whether HAT activity plays a role in tumor suppressor activity of BRCA2. We plan to identify the factors that associate with BRCA2 and study the role of these protein-protein interactions in the biochemical and biological properties of BRCA2. We intend test whether transcriptional activation function has any role in the tumor suppressor activity of BRCA2. We intend to identify the BRCA2 target genes and study (in future) their role in the tumor suppressor activity of BRCA2.

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INTRODUCTION:

Alterations in BRCA1 and BRCA2 tumor suppressor genes have been shown to account up to 90% of familial breast cancers and also a proportion of familial ovarian cancers. Since loss of wild type BRCA2 allele in heterozygous carriers results in breast and ovarian cancers, BRCA2 is regarded as a tumor suppressor gene. Interestingly, both BRCA2 and BRCA1 are expressed coordinately and also in a cell cycle-dependent manner. Two potential functions of BRCA2 were proposed which includes a role in DNA repair and in the regulation of transcription.

Interestingly, BRCA1 proteins were shown to have transcriptional activation function and also shown to be associated with RNA polymerase. These results suggest that both BRCA2 and BRCA1 proteins may function as transcriptional factors and have a role in the regulation of transcription. Recent studies on enzymes responsible for histone acetylation revealed that some of the transcriptional factors function as histone acetyl transferases. Since BRCA2 showed transcriptional activation properties, we tested whether BRCA2 functions as a histone acetyltransferase. Our recent results suggested that BRCA2 is associated with histone acetyl transferase (HAT) (1). As mentioned above, it is possible that BRCA2 also recruit other transcriptional factors which themselves function as HAT proteins. Identification of these recruitment partners will provide in sights into the molecular mechanism of growth and tumor suppressor function of BRCA2. Taking into consideration of the presence of transcriptional activation domain in BRCA2 and also having intrinsic HAT activity, it is not unreasonable to suggest that BRCA2 is a transcriptional factor. This proposal is aimed to strengthen this notion of BRCA2. Our recent finding that BRCA2 interacts with CBP supports this conclusion. It is possible that BRCA2 and CBP/p300 and other factors acetylate histones bound at specific promoters through cooperative acetylation and this could be a mechanism by which BRCA2 activates genes responsible for growth inhibition and differentiation. Therefore, identification of target genes of BRCA2 and its associating factors may provide a clue to the tumor suppressor function of BRCA2. Because of its large size, it is possible that BRCA2 has multicellular functions which include DNA repair, transactivation, HAT activity etc. which may play a role in tumor suppressor function.

BODY:

Several transcriptional factors (CREB, ACTR, SRC-1, P/CAF, TAF 250, nuclear hormone receptors etc.) have been demonstrated to interact with CBP (2). Some of these transcriptional factors (ACTR, SRC-1, P/CAF. TAF 250 etc.) were found to have intrinsic HAT activity and recruit other transcriptional factors (such as CBP/p300 etc) which have intrinsic HAT activity to provide a cooperative regulatory effect on gene expression (3). Since BRCA2 is a transcriptional factor, we hypothesized that BRCA2 may interact with CBP and other factors and acetylate histones bound at specific promoters through cooperative acetylation and this could be a mechanism by which BRCA2 activates genes responsible for growth inhibition and differentiation. To test this hypothesis, we have studied the interaction of BRCA2 and CBP by GST pull-down assay. Briefly, we have cloned BRCA2 cDNA fragment into pcDNA-3 vector and linearized the vector with appropriate restriction enzyme. We have used this linearized expression vector carrying BRCA2 cDNA as a template and carried out in vitro transcription and

translation in the presence of ^{35} S-methionine. This radiolabelled BRCA2 protein was incubated with beads containing GST-CBP-1, GST-CBP-2 and GST respectively. CBP-1 (a 451-662) and CBP-2 (1680-1891) represent amino and carboxy-terminal domains that were shown to interact with a variety of transcriptional factors. CBP-1 was shown to interact with CREB, Sap-1a, c-Myb and c-Jun etc where as CBP-2 is shown to interact with E1A, P/CAF, c-Fos, MyoD etc. In vitro translated [S]^{35}-Methionine labeled BRCA2 (a 1-500) was bound to GST-CBP-2 but not to GST-CBP-1 and GST itself. These preliminary results suggested that BRCA2 interacts with CBP *in vitro*. We proposed to confirm these results. As per the proposal we repeated these experiments twice and confirmed that BRCA2 interacts with CBP. Our recent results suggest that BRCA2 appears to be an unstable enzyme that lose irreversibly HAT activity with storage. Such rapid and irreversible loss of HAT activity was also observed in the case of other HAT enzymes namely GCN5, PCAF etc. We have also observed such loss of HAT activity with CBP on storage.

Our preliminary results suggested that BRCA2 and BRCA2a interacts with p53. We repeated these experiments using GST pull down assay and confirmed that indeed p53 directly interacts with the BRCA2 and BRCA2a. We repeated GST pull down assays and confirmed that p53 interacts with aminoterminal region of BRCA2 (aa 189-500).

In order to determine the BRCA2 domain responsible for interaction with p53, we have made carboxy-terminal deletion of BRCA2 and BRCA2a and expressed in bacteria and purified by affinity column. We repeated our preliminary results and confirmed that p53 showed very weak binding to carboxyl-truncated BRCA2 and BRCA2a suggesting that amino acid 189-500 is essential for interaction with p53.

Since BRCA2 physically interacts with p53, we tested what effect BRCA2 will have on the transcriptional activation properties of p53. pcDNA-BRCA2 or pcDNA BRCA2a either alone or together with p53 was cotransfected into Saos-2 cells (p53 null Osteosarcoma cells) together with p53 reporter plasmid (pG13-CAT). Our results suggest that the expression of BRCA2 and BRCA2a had no effect on the expression of p53-specific reporter in the absence of exogenous wild type p53. As expected, expression of wild type p53 resulted in transcriptional activation of p53 reporter plasmid (pG13-CAT) (a gift from Dr. Vogelstein). However, co-transfection of BRCA2 (or BRCA2a) and p53 resulted in a dramatic increase in CAT activity of pG13-CAT suggesting that BRCA2 and BRCA2a cooperates with p53 in the transcriptional activation of p53 reporter plasmid. Therefore, our preliminary results suggest that BRCA2 and BRCA2a function as transcriptional co-factor of p53. We proposed to confirm these results. As per the proposal, we have repeated these experiments three times and confirmed that BRCA2 co-operates with p53 in trans activation function. These results may shed new clues in understanding the function of BRCA2.

Interestingly, both BRCA2 and BRCA1 proteins were shown to have transcriptional activation domains and also shown to be associated with RNA polymerase suggesting that these proteins may function as transcriptional factors and have a role in the regulation of transcription. The expression pattern, common protein-protein interactions and the common breast cancer phenotype observed in the patients that carry mutations in these genes suggest that BRCA1 and BRCA2 may function in a similar, if not, same pathway. We have identified several common key

proteins that interact with BRCA2 and BRCA1 and at present we are assessing their role in the biological function of BRCA2. We have also identified a new molecular target of action of breast cancer (4) (In collaboration with Dr. Veena Rao). Our results demonstrate that BRCA1 targets Elk-1 (previously discovered by us) to show growth suppressor properties (4). These results show for the first time a link between the growth suppressive function of BRCA1 and signal transduction pathway involving Elk-1. It will be interesting to see whether Elk-1 plays a similar role in growth suppressive function of BRCA2.

Since the expression of BRCA1 and BRCA2 gene products are coordinately regulated and their expression peaks at the G1/S boundary of the cell cycle, BRCA1 and BRCA2 may show similar functional properties in spite of the fact that they show no major sequence homology. Since BRCA1 interacts with E2F, cyclins and CDKs (which are known to regulate the cell cycle), we hypothesized that BRCA2 may be regulating the cell cycle by interacting with these proteins. To test this hypothesis, we have carried out GST- pull down assays to study the interaction of BRCA2 with E2F, cyclins and CDKs. Our results demonstrate that both BRCA2 and BRCA2a associate with transcriptional factor E2F, cyclins and cyclin dependent kinases (CDKs). We have localized the domains of BRCA2 that is responsible for these protein-protein interactions. Patients with mutations in these domains may have impaired binding of BRCA2 to E2F-1, cyclins/CDKs, resulting in a loss of the negative regulation of cell cycle/cell proliferation and predisposition to breast cancers.

KEY RESEARCH ACCOMPLISHMENTS:

BRCA2 and BRCA2a interact with CBP carboxyterminal region. BRCA2 and BRCA2a also interact with p53 in vitro. We have confirmed that p53 interacts with the aminoterminal region of BRCA2 (aa 189-500). Our results show that BRCA2 cooperates with tumor suppressor p53 in the transcriptional activation function. Our results also demonstrate that there may be a link between the growth suppressive function of BRCA1/BRCA2 and signal transduction pathway involving Elk-1.

REPORTABLE OUTCOMES:

1. Siddique, H., Zou, J.P., Rao, V.N., and Reddy, E.S.P. The BRCA2 is a histone acetyltreansferase. Oncogene. *16*: 2283-5, 1998.

2. Chai, Y.L., Cui, J., Shao, N., **Reddy, E.S.P.**, and Rao, V.N. The second BRCT domain of BRCA1 proteins interacts with p53 and stimulates transcription from the $p21^{WAF1/CIP1}$ promoter. **Oncogene** 18, 263-268, 1999.

3. Chai, Y.L., Cui, J., Shao, N., **Reddy, E.S.P.**, and Rao, V.N. The second BRCT domain of BRCA1 proteins interacts with p53 and stimulates transcription from the $p21^{WAF1/CIP1}$ promoter. **Oncogene** 18, 263-268, 1999.

4. Zou, J.P., Hirose, Y., Siddique, H., Rao, V.N. and Reddy, E.S.P. Structure and expression of variant BRCA2a lacking the transactivation domain. Oncology Reports. *6*: 437-440, 1999.

5. Chai, Y.L., Cui, J., Chipitsyna, G., Liao, B., Liu, S., Yezdani, M., Reddy, E.S.P. and Rao, V.N. c-Fos oncogene regulator Elk-1 interacts with BRCA1 splice variants BRCA1a/1b and enhances BRCA1a/1b mediated growth suppression in breast cancer cells. Oncogene, 20, 1357-1367, 2001.

ABSTRACTS:

1. Zou, J.P., Siddique, H., Lee, L., Rao, V.N. and **Reddy, E.S.P.** BRCA2 and the variant BRCA2a lacking the transactivation domain interact with E2F, cyclins and CDKs. Poster Presentation at the Drexel and MCP Hahnemann Sigma Xi Research Day, May 6, 1999.

2. Siddique, H., Zou, J.P., Lee, L., Rao, V.N. and **Reddy, E.S.P.** BRCA2 functions as a transcriptional co-factor of p53. Poster Presentation at the Drexel and MCP Hahnemann Sigma Xi Research Day, May 6, 1999.

3. Chai, Y.L., Shao, N., Chipitsyna, G., **Reddy, E.S.P.** and Rao, V.N. BRCA1 Splice Variants, Phosphorylation, Tumor Suppression, Apoptosis and Protein-Protein Interaction. Poster Presentation at the Drexel and MCP Hahnemann Sigma Xi Research Day, May 6, 1999.

4. Shao, N., Chai, Y.L., Chipitsyna, G., Liao, B., Liu, S., **Reddy, E.S.P.** and Rao, V.N. Elk-1, a c-fos regulator and ΔElk-1 induce apoptosis in breast cancer cells. Poster Presentation at the Drexel and MCP Hahnemann Sigma Xi Research Day, May 6, 1999.

5. Fujimura, Y., Lee, L., Rao, V.N. and **Reddy, E.S.P.** Aberrant Fusion Proteins Target Transcriptional Co-Activator CBP-p300. Poster Presentation at the Pan-Pacific Lymphoma Conference, July, 1999.

6. Chai, Y.L., Shao, N., Chipitsyna, G., **Reddy, E.S.P.** and Rao, V.N. BRCA1 Splice Variants, Phosphorylation, Tumor Suppression, Apoptosis and Protein-Protein Interaction. Poster Presentation at the Pan-Pacific Lymphoma Conference, July, 1999.

7. Siddique, H., Lee, L., Rao, V.N. and **Reddy, E.S.P.** Aberrant EWS-PEA3/E1AF Protein is a Transcriptional Activator. Poster Presentation at the Drexel and MCP Hahnemann Sigma Xi Research Day, May 2, 2000.

8. Liu, F., Fujimura, Y., Hirose, Y., Zou, J.P. Lee, L., Rao, V.N. and **Reddy, E.S.P.** Structure and Function of novel isoforms of PTEN involved in several human cancers. Poster Presentation at the Drexel and MCP Hahnemann Sigma Xi Research Day, May 2, 2000.

9. Cui, J., Chai, Y.L., Shao, N., Liao, B., Liu, Shuang, Chipitsyna, G., Yezdani, M., **Reddy, E.S.P.** and Rao, V.N. Functional dissection of BRCA1 proteins and their role in breast cancer. Poster Presentation at the Drexel and MCP Hahnemann Sigma Xi Research Day, May 2, 2000.

10. Chai, Y.L., Cui, J., Chipitsyna, G., Liao, B., Liu, S., Yezdani, M., **Reddy, E.S.P.** and Rao, V.N. Identification of a new molecular target of action of BRCA1 in breast cancer, Poster Presentation at the Drexel and MCP Hahnemann Third annual Research Day, May 1, 2001.

11. Fujimura, Y., Siddique, H., Rao, V.N. Ramugounder, R., Liu, F., Jian-Ping Zou, Senadhi, V. and **Reddy, E.S.P.** The aberrant EWS-ATF-1 fusion proteins involved in clear cell sarcoma target the transcriptional coactivator CBP/p300. Poster Presentation at the Drexel and MCP Hahnemann Third annual Research Day, May 1, 2001

12. Fujimura, Y., Siddique, H., Rao, V.N. Ramugounder, R., Liu, F., Jian-Ping Zou, Senadhi, V. and **Reddy, E.S.P.** EWS-ATF-1 chimeric protein in soft tissue clear cell sarcoma associates with CREB-Binding Protein and interferes with p53-mediated trans-activation function. Poster Presentation at the Drexel and MCP Hahnemann Discovery 2001 Research meeting. Oct 4, 2001.

13. Chai, Y.L., Chipitsyna, G.,N. Rath, M. Steans, D. Rukstalis, Reddy, E.S.P. and **Rao, V.N.** BRCA1 splice variant BRCA1a functions as a growth/tumor suppressor of human breast, ovarian and prostate cancer cells. Poster Presentation at the Drexel and MCP Hahnemann Third annual Research Day, 2002.

14. BRCA2 and the variant BRCA2a lacking the transactivation domain functions as a transcriptional coactivator of p53 and interact with E2F, cyclins and CDKs. Siddique, H.,Zou, J.P., Lee, L., Ramugounder, R., Liu, F., Fujimura, Y., **Rao, V.N.** and Reddy, E.S.P. Era of Hope, Department of Defense Breast Cancer Research Program meetimg, Orlando, Florida, 2002.

CONCLUSIONS:

Our research on this project suggests that BRCA2 and BRCA2a interact with CBP carboxyterminal region suggesting that BRCA2 and BRCA2a may undergo posttranslational modification. These posttranslational modifications may have profound effect on the function of BRCA2 and BRCA2a. Therefore, identifying what type of translation modification takes place as a result of protein-protein interaction of BRCA2/BRCA2a and CBP is important for future work. Understanding the effect protein-protein interaction of p53 and BRCA2/BRCA2a on p53-mediated transcriptional activity may provide new clues to the role of BRCA2 in tumor growth suppressor function.

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3. Leo, C. and Chen, J. D. The SRC family of nuclear receptor coactivators, Gene. *245:* 1-11, 2000.

4. Chai, Y.L., Cui, J., Chipitsyna, G., Liao, B., Liu, S., Yezdani, M., Reddy, E.S.P. and Rao, V.N. c-Fos oncogene regulator Elk-1 interacts with BRCA1 splice variants BRCA1a/1b and enhances BRCA1a/1b mediated growth suppression in breast cancer cells. Oncogene, 20, 1357-1367, 2001.

APPENDICES:

1. Siddique, H., Zou, J.P., Rao, V.N., and Reddy, E.S.P. The BRCA2 is a histone acetyltreansferase. Oncogene. *16*: 2283-5, 1998.

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Oncogene (1998) 16, 2283–2285

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SHORT REPORT

The BRCA2 is a histone acetyltransferase

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Patients carrying mutations in BRCA1 or BRCA2 tumor suppressor genes have shown to have high risk in developing breast and ovarian cancers. Two potential functions of BRCA2 were proposed which includes role in the regulation of transcription and also in DNA repair. Forty-five-amino acid region encoded by exon 3 of BRCA2 was shown to have transcriptional activation function. Recent studies of the several enzymes involved in acetylation and deacetylation of histone residues have revealed a possible relationship between gene transcriptional activation and histone acetylation. Since BRCA2 appear to function as a transcriptional factor, we have tested for Histone acetyl transferase (HAT) activity of BRCA2. Here, we present evidence that BRCA2 has intrinsic HAT activity, which maps to the amino-terminal region of BRCA2. Our results demonstrate that BRCA2 proteins acetylate primarily H3 and H4 of free histones. These observations suggest that HAT activity of BRCA2 may play an important role in the regulation of transcription and tumor suppressor function.

Keywords: BRCA2; histone acetyl transferase; protein-protein interaction; tumor suppressor

Alterations in *BRCA1* and *BRCA2* tumor suppressor genes have been shown to be involved in 90% of familial breast cancers (Newman *et al.*, 1988; Miki *et al.*, 1994; Easton *et al.*, 1993; Wooster *et al.*, 1994; Wooster and Stratton, 1995). Recent studies revealed that both BRCA1 and BRCA2 are involved in ovarian and prostate cancers. Interestingly, BRCA2 was found to be more associated with male breast cancer compared to BRCA1 (Wooster *et al.*, 1994). Patients with BRCA2 mutations were also found to be at a higher risk with a variety of other cancers including carcinomas of pancreas, prostate and colon (Thorlacius *et al.*, 1996; Phelan *et al.*, 1996; Gudmundsson *et al.*, 1995; Tonin *et al.*, 1995). The BRCA2 gene is composed of 27 exons and encodes a protein of 3418 amino-acids with no significant homology to any known protein (Wooster *et al.*, 1995; Bork *et al.*, 1996). BRCA2 and BRCA1 proteins have been shown to interact with Rad 51 which suggests that they play a role in DNA repair (Scully *et al.*, 1997; Sharan *et al.*, 1997; Zhang *et al.*, 1998). BRCA1 was also shown to induce apoptosis suggesting that BRCA proteins may play a role in the regulation of apoptosis of cells (Shao *et al.*, 1996; Rao *et al.*, 1996). It remains to be seen whether BRCA2 plays a similar role in apoptosis.

Interestingly, both *BRCA1* and *BRCA2* gene products are regulated in a cell cycle-dependent manner and have a potential transactivation function (Rajan *et al.*, 1996;' Vaughn *et al.*, 1996; Chapman and Verma, 1996; Monteriro *et al.*, 1996; Milner *et al.*, 1997; Wang *et al.*, 1997; Cui *et al.*, 1998a). Recently, we have shown that BRCA1 proteins interact with transcriptional co-activator CBP suggesting that BRCA1 has a role in the regulation of transcription (Cui *et al.*, 1998b). Exon 3 of BRCA2 was found to have weak homology with transcriptional factor c-*jun* and also shown to activate transcription in mammalian cells (Milner *et al.*, 1997). These results suggest that BRCA2r has a role in the regulation of gene expression.

Recent studies of the several enzymes involved in acetylation and deacetylation of histone residues have revealed a possible relationship between gene transcriptional activation and histone acetylation (Brownell *et al.*, 1996; Parthun *et al.*, 1996; Yang *et al.*, 1996; Orgyzko *et al.*, 1996; Mizzen *et al.*, 1996; Roth and Allis, 1996; Wade and Wolffe, 1997; Pazin and Kadonaga, 1997; Wolffe, 1997). This view is supported by the identification of Histone acetyl transferase (HAT) activity associated with several transcription factors including p300/CBP, GCN5-related factors, p/CAF, SRC-1 and TAF_{II} 250. These results suggest that some transcriptional activators operate by disrupting the nucleosomal structure through acetylation of histones leading to the activation of gene expression.

Here, we report for the first time that the amino-terminal region of BRCA2 has intrinsic HAT activity from which it may be inferred that BRCA2 joins the above list of transcriptional activators/factors that possess HAT activity. This intrinsic BRCA2-HAT activity may play a key role in the tumor suppressor function of BRCA2.

Recently, we have cloned an alternatively spliced isoform, BRCA2a. This variant BRCA2a lacks a transcriptional activation domain (exon 3) as a result of alternative splicing (our unpublished results). In order to test the HAT activity of BRCA2, we have expressed the amino-terminal region of BRCA2 (aa 1–500) and its isoform BRCA2a (aa (1–18)-(105–500)) as GST-fusion proteins in bacteria by cloning appropriate BRCA2 cDNA fragments into a GST expression vector (Our unpublished results). Purified recombinant proteins of BRCA2 and BRCA2a were assayed for histone acetyl transferase activity. Amino-terminal domains of both BRCA2 and BRCA2a clearly demonstrated histone acetyl transferase activity (Figure 1). Control samples where BRCA2 or BRCA2a was replaced with bovine serum albumin (BSA) showed no

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significant HAT activity. Similar control experiments where histones were replaced by BSA (lysine rich nonhistone protein) also failed to show significant acetyl transferase activity. This suggests that BRCA2 proteins show specific acetyl transferase activity to histones (Figure 1). Therefore, we conclude that BRCA2 *per se* is a histone acetyl transferase. Since the amino-terminal region of BRCA2 and BRCA2a show HAT activity, we conclude that the exon 3 (aa 18–105) responsible for the transactivation function of BRCA2 is not needed for HAT activity function. These results suggest that the transactivation and HAT functional domains of BRCA2 do not overlap with each other (Figure 2).

In order to determine which histones are acetylated by BRCA2 proteins, we have carried out HAT assay with free core histones and analysed the resulting products by SDS-polyacrylamide gel electrophoresis followed by fluorography. Our results demonstrate that BRCA2 proteins acetylated primarily H3 and H4 of free histones (Figure 3). We have also confirmed these results using individual free histones (data not shown).

In order to determine the HAT activity associated with BRCA2 *in vivo*, we have carried out immunoprecipitation HAT assay. Immunoprecipitation of BRCA2 from whole cell extracts was tested for acetyl transferase activity. Our results demonstrate that immunoprecipitated BRCA2 carries acetylase activity specific for histones (Figure 4). These *in vitro* and *in vivo* results support the conclusion that BRCA2 has intrinsic HAT activity. It is conceivable that as in the case of CBP/p300 (which shows intrinsic HAT activity), transcriptional activators recruit BRCA2 and utilize its intrinsic HAT activity for their transcriptional activation properties. It is also possible that BRCA2 also in turn recruits other factors (like p/CAF, p300/CBP) that possess distinct HAT activity and thereby disrupt the nucleosomal structure through their cooperative HAT activity. This results in the activation of gene expression interacts with CBP both *in vitro* and *in vivo*. Therefore, it is tempting to speculate that the target genes of BRCA2 play key roles in growth inhibition, differentiation and apoptosis. Identification of these genes may provide clues to the role of BRCA2 in neoplasia. Because of its large size, it is conceivable that BRCA2 has multicellular functions which include DNA repair, transcriptional activation, HAT etc. It is possible that BRCA2/Rad 51 Complex may use HAT activity to disrupt the nucleosomal structure to recognize

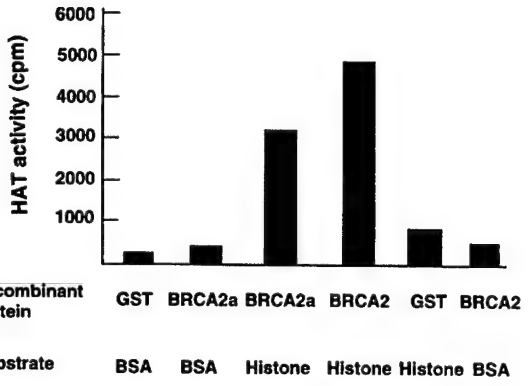

Figure 1 BRCA2 has intrinsic HAT activity. The amino-terminal region of BRCA2 (aa 1–500) and BRCA2a ((1–18)-(105–500)) were expressed as GST fusion proteins in bacteria and subsequently purified. HAT assays were caried out as described but with slight modification (Bannister and Kouzarides, 1996; Herrera *et al*., 1997). Approximately 50–100 ng of GST-fusion proteins of BRCA2 and BRCA2a were used to acetylate 15 μg of core histones (Boehringer Mannheim) in the presence of [^{3}H]acetyl CoA

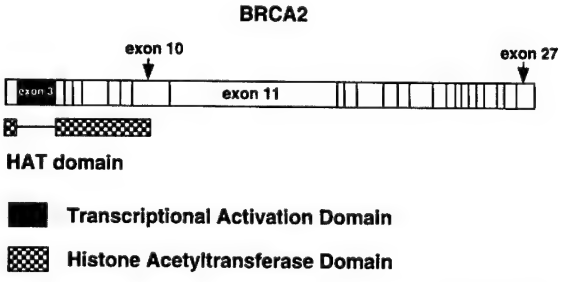

Figure 2 Schematic representation of the functional domains of BRCA2

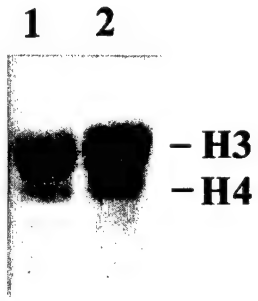

Figure 3 Acetylation profile and Substrate specificity of BRCA2 and BRCA2a. Recombinant BRCA2a (aa 1–500) (lane 1) and BRCA 2 ((1–18)-(105–500)) (lane 2) were incubated with core histones as described above. [^{14}C]acetylated histones were separated on SDS–PAGE and detected by autoradiography

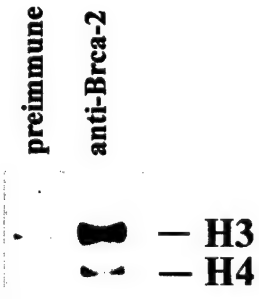

Figure 4 BRCA2 antibodies immunoprecipitate HAT activity. Immunoprecipitations (IP) were performed from NIH3T3 whole cell extract with either anti-BRCA2 (Santa Cruz) antibody or pre-immune serum. These IPs were tested for their ability to acetylate free histones as described above. Pre-immune serum served as a negative control

damaged DNA for DNA repair. Patients with mutations in HAT and/or transactivation domains of BRCA2 may show a loss of gene expression whch are critical for growth inhibition and differentiation and result in a subset of familial breast and prostate cancers. One can use BRCA2-HAT assay for screening patients with BRCA2 mutations.

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SHORT REPORT

The second BRCT domain of BRCA1 proteins interacts with p53 and stimulates transcription from the p21$^{WAF1/CIP1}$ promoter

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Inherited mutations in the breast and ovarian cancer susceptibility gene BRCA1 are associated with high risk for developing breast and ovarian cancers. Several studies link BRCA1 to transcriptional regulation, DNA repair, apoptosis and growth/tumor suppression. BRCA1 associates with p53 and stimulates transcription in both p53 dependent and p53-independent manners. BRCA1 splice variants BRCA1a (p110) and BRCA1b (p100) associates with CBP/p300 co-activators. Here we show that BRCA1a and BRCA1b proteins stimulate p53-dependent transcription from the p21$^{WAF1/CIP1}$ promoter. In addition, the C-terminal second BRCA1 (BRCT) domain is sufficient for p53 mediated transactivation of the p21 promoter. Previous studies emphasized the importance of the BRCT domain, which shows homology with p53 binding protein (53BP1), in transcriptional activation, growth inhibition and tumor suppression. Our findings demonstrate an additional function for this domain in protein–protein interaction and co-activation of p53. We also found that BRCA1a and BRCA1b proteins interact with p53 *in vitro* and *in vivo*. The p53 interaction domain of BRCA1a/1b maps, *in vitro*, to the second BRCT domain (aa 1760–1863). The BRCT domain binds to the central domain of p53 which is required for sequence specific DNA binding. These results demonstrate for the first time the presence of a second p53 interaction domain in BRCA1 proteins and suggests that BRCA1a and BRCA1b proteins, like BRCA1, function as p53 co-activators. This BRCT domain also binds *in vitro* to CBP. These results suggest that one of the mechanisms by which BRCA1 proteins function is through recruitment of CBP/p300 associated HAT/FAT activity for acetylation of p53 to specific promoters resulting in transcriptional activation.

Keywords: BRCA1; BRCA1a; BRCA1b; p53; p21$^{WAF1/CIP1}$; BRCT domain; CBP/p300

BRCA1, a familial breast and ovarian cancer susceptibility gene, is linked to 45% of the families with inherited breast cancers and about 90% of families with inherited breast and ovarian cancers (Miki *et al.*, 1994; Easton *et al.*, 1995; Ford *et al.*, 1995). BRCA1 encodes a protein of 1863 amino acids. The unique amino-terminal zinc finger domain interacts with BARD1 (Wu *et al.*, 1996), E2F transcriptional factors, cyclins and cyclin-dependent kinases (Wang *et al.*, 1997). Two, tandem carboxy-terminal BRCT domains (BRCA1 C-terminal domain (Koonin *et al.*, 1996; Bork *et al.*, 1997; Callebaut and Mornon, 1997) are involved in transcription activation, growth inhibition and tumor suppression (Chapman and Verma, 1996; Monteiro *et al.*, 1996; Rao *et al.*, unpublished results; Holt *et al.*, 1996; Thompson *et al.*, 1995; Rao *et al.*, 1996; Humphrey *et al.*, 1997). The BRCT domains of BRCA1 are targets for cancer associated mutations (Couch and Weber, 1996) and are conserved evolutionary (Szabo *et al.*, 1996). Several recent reports have shown moderate homology between the BRCT domains of BRCA1 and the C-terminal region of p53-binding protein (5BP1), the yeast RAD9 protein and multiple proteins involved in cell cycle checkpoint functions responsive to DNA damage (Koonin *et al.*, 1996; Bork *et al.*, 1997; Callebaut and Mornon, 1997). Recently, we and others showed that the N- and C-terminal regions of BRCA1, BRCA1a and BRCA1b proteins activate transcription when fused to GAL4 DNA binding domain (Cui *et al.*, 1998a; Chapman and Verma, 1996; Monteiro *et al.*, 1996), and associate with RNA polymerase II holoenzyme (Scully *et al.*, 1997a) and CBP/P-300 co-activator (Cui *et al.*, 1998b), suggesting a potential role for BRCT domains in the regulation of transcription. The multiple functions of BRCA1 protein(s) include growth/tumor suppression (Holt *et al.*, 1996; Thompson *et al.*, 1995; Rao *et al.*, 1996; Humphrey *et al.*, 1997), induction of apoptosis (Shao *et al.*, 1996), cell cycle regulated expression, DNA repair and the maintenance of genomic stability (Lane *et al.*, 1995; Marquis *et al.*, 1995; Scully *et al.*, 1997b; Brugarolas and Jacks, 1997). The BRCA1 protein physically associates, both *in vitro* and *in vivo*, with p53 tumor suppressor gene and stimulates p53-dependent transcription from the p21$^{WAF1/CIP1}$ and bax promoters (Ouchi *et al.*, 1998; Zhang *et al.*, 1998). BRCA1 has also been shown to transactivate expression of the cyclin-dependent kinase inhibitor p21 in a p53-independent manner (Somasundaram *et al.*, 1997), suggesting that one of the mechanisms by which BRCA1 regulates cell cycle and suppresses growth is through the induction of p21 (Somasundaram *et al.*, 1997).

p53 is a tumor suppressor protein that is altered in numerous human malignancies including colon, lung, breast, ovary and several others (Hollstein *et al.*, 1991). The p53 protein has been implicated in a number of functions, including cell cycle regulation, response to DNA damage, signal transduction, cellular differentiation and activation and repression of transcription (Ko

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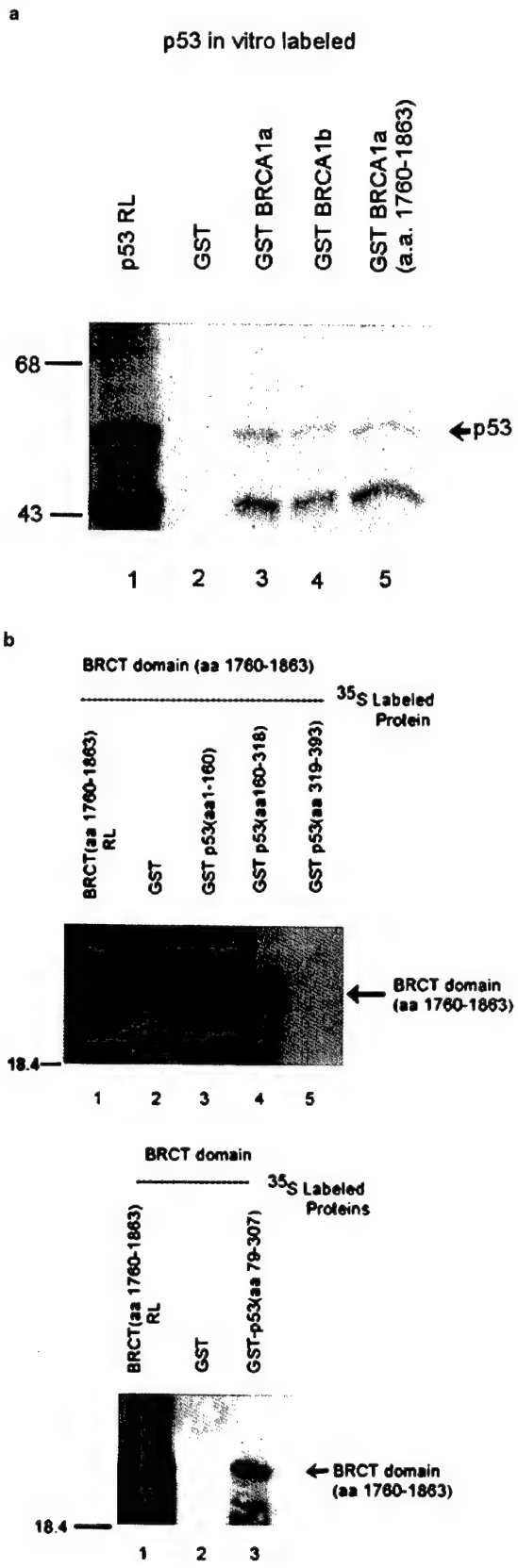

Figure 1 Direct physical association of p53 with BRCA1a, BRCA1b and the second BRCT domain of BRCA1, *in vitro*. (**a**) GST, GST-BRCA1a, GST-BRCA1b and GST-BRCT domain (amino acids 1760–1863) proteins were expressed, purified on beads and incubated with *in vitro* translated ^{35}S-methionine-labeled p53 and subjected to GST-pull down assay as described previously (Wang *et al*., 1997; Cui *et al*., 1998b). In lane 1, 1/20th of the *in vitro* translated protein used for binding was loaded directly. The second protein band seen in lane 1 represents polypeptide arising as a result of initiation at the internal methionine codon or premature termination. (**b**) BRCT domain (amino acids 1760–1863) binds to the central domain of p53 (amino acids 99–307). GST, GST-p53 (amino acids 1–160), GST-p53 (amino acids 160–318), GST-p53 (amino acids 319–393) and GST-p53 (amino acids 99–307) proteins were expressed, purified on beads and incubated with *in vitro* translated ^{35}S-methione-labeled BRCT domain (amino acids 1760–1863) and subjected to GST-pull down assay as described above

and Prives, 1996). The p53 protein contains distinct functional domains: a transcriptional activation domain at the amino terminal (residues ~1–70); a SH3-like domain (residues ~61–94); a central sequence-specific DNA binding domain (residues ~102–392); a nonspecific DNA-binding domain at the carboxy terminal region (residues ~320–393) which contains a tetramerization domain (Stürzbecher *et al*., 1992); and a basic domain (Ko and Prives, 1996 and references therein).

The presence of a common BRCT domain between BRCA1 and p53 binding protein 53BP1 (Iwabuchi *et al*., 1994), which is also present in splice variants BRCA1a and BRCA1b (Cui *et al*., 1998a), led us to investigate whether BRCA1 proteins associate with p53, the universal tumor suppressor, as speculated earlier by others (Bork *et al*., 1997; Koonin *et al*., 1996). To determine whether intact BRCA1a/1b and a deletion mutant (containing the second BRCT domain aa 1760–1863) bind with p53, *in vitro* binding was performed with full length wild type human p53 protein. The *in vitro* translated ^{35}S-methionine-labeled p53 protein was passed through GST-BRCA1a, GST-BRCA1b, GST-BRCT domain (residues 1760–1863) and a GST control column as described previously (Wang *et al*., 1997; Cui *et al*., 1998b). p53 was found to bind specifically to GST-BRCA1a, GST-BRCA1b, GST-BRCT domain (residues 1760–1863) but not to GST (Figure 1a, lanes 3, 4 and 5). The region of BRCA1/1a/1b extending from aa residues 1760–1863, which also maps to the minimum transactivation domain, was found to be sufficient for interaction with p53. Recently, the N-terminal region of exon 11 (aa 224–500) was shown to interact with the C-terminal domain (aa 300–393) of p53 (Zhang *et al*., 1998). Interestingly, majority of exon 11 (aa 263–1365) is lost in BRCA1a and BRCA1b (Cui *et al*., 1998a; Rao, unpublished results) but they still bind to p53. Our results demonstrate for the first time a second p53 interaction domain in the second BRCT domain of BRCA1 protein. To map the region of p53 which binds to the BRCT domain, a series of p53 deletion mutants expressed as GST fusion proteins (generous gifts from Drs Shenk and Wiman) were incubated with *in vitro* translated BRCT domain (aa 1760–1863) protein and subjected to GST-pull down assay. The results show that BRCT domain binds to the central region but not to the C-terminal region (amino acids 319–393) (Figure 1b, top panel lanes 3 and 4, bottom panel lane 3). These results suggest that the BRCT domain of BRCA1 protein binds to the central domain of p53 similar to 53BP1, 53BP2 and SV40 large tumor antigen. This central domain is responsible for site

specific DNA binding and contains mutations that are found commonly in tumors. Since BRCA1a, BRCA1b and the BRCT domain interact *in vitro* with p53, we investigated the effects of BRCA1a/1b and BRCT on p53 independent and p53 dependent transcription. Since both p53 and BRCA1 can transactivate the p21 promoter (El-Deiry *et al*., 1995; Somasundaram *et al*., 1997), we studied the effects of BRCA1a and BRCA1b on the p21 promoter. pcDNA-BRCA1a, pcDNA-BRCA1b or pcDNA-BRCT (1760–1863), both alone and in the presence of pSGp53 were co-transfected into COS-7 cells together with a natural genomic p21 promoter CAT vector (a generous gift from Dr Volgelstein) that contains the p53 response element (El-Deiry *et al*., 1995). Neither BRCA1a, BRCA1b nor BRCT domain (residues 1760–1863) activated the p21 promoter in the absence of exogenous p53 (Figure 2), but all stimulated the p21 promotor in the presence of p53 (Figure 2). These results suggest that BRCA1a and BRCA1b proteins function as co-activators of p53 and the C-terminal second BRCT domain of BRCA1/1a/1b proteins is sufficient for binding to p53 and stimulating its target gene activation. As mentioned earlier, several groups have shown that BRCA1 regulates p21 through both p53-dependent and p53-independent mechanisms (Ouchi *et al*., 1998; Zhang *et al*., 1998; Somasundaram *et al*., 1997). Since BRCA1a/1b lack the N-terminal region of exon 11 which was also shown to interact with p53, we speculate that BRCA1a/p110 and BRCA1b/p100 can regulate p21 transcription only through a p53-dependent mechanism. All these results taken together suggest a role for p53 in the growth arrest, tumor suppression and apoptosis-inducing functions of BRCA1 proteins.

To study these associations in a physiologically relevant environment, cell extracts from human breast cancer cells CAL-51 were immunoprecipitated with anti-BRCA1 polyclonal antibody and as a negative control, normal rabbit IgG. Subsequent Western blot analysis with anti-p53 monoclonal antibody revealed p53 in the anti-BRCA1 immunoprecipitates (Figure 3a, lane 2), and nuclear extract (Figure 3a, lane 3), but not in the control immunoprecipitate (Figure 3a, lane 1). The anti-BRCA1 antibody C-20 detects both BRCA1a/1b proteins by immunoprecipitation and Western blot analysis (Figure 3b). All these results suggest that BRCA1a/1b proteins associate with p53 *in vivo*.

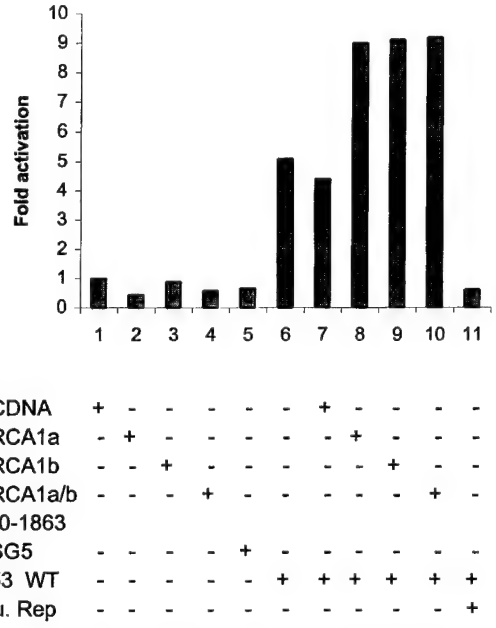

Figure 2 The second BRCT domain of BRCA1/1a/1b is sufficient for p53 dependent transactivation of the p21$^{WAF1/CIP1}$ promoter. COS-7 cells were co-transfected with 1 μg of human p21 promoter CAT 'A' or 'E' construct (El-Deiry *et al*., 1995) and 1 μg of expression plasmids (pcDNA BRCA1a, pcDNA-BRCA1b, pcDNA-BRCT amino acids 1760–1863 and pSGp53 wild type) as described previously (Cui *et al*., 1998a,b). Total DNA was kept constant at 20 μg. The CAT activity shown represents fold activity compared with pcDNA vector alone. The activity of the vector is normalized to a value of one. The experiments were repeated at least four times. Mu-Rep represents p21 promoter construct 'E' (El-Deiry *et al*., 1995) which lacks p53 binding sites. The results of CAT assay were quantitated using a Fuji phosphoimager

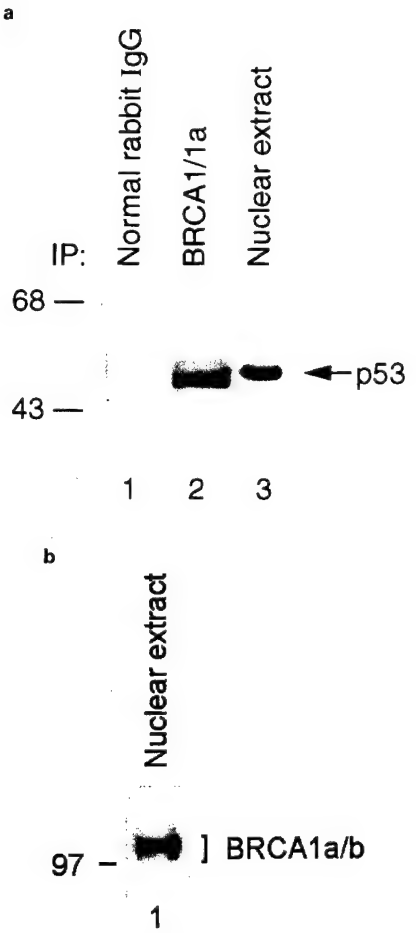

Figure 3 *In vivo* interaction of p53 with BRCA1a/1b proteins. (**a**) BRCA1 proteins were immunoprecipitated by anti-BRCA1 antibody from cell extracts of human breast cancer cell line CAL51, separated on 10% SDS–PAGE analysis and Western blotted with anti-p53 monoclonal antibody. Lane 1, immunoprecipitation by normal rabbit IgG (negative control), lane 2, immunoprecipitation by anti-BRCA1 antibody, lane 3 A431 nuclear extract (positive control for p53). (**b**) Western blot analysis of nuclear extract from A431 cells using anti-BRCA1 antibody after immunoprecipitation with anti-BRCA1 antibody was done as described (Cui *et al*., 1998b). The protein bands shown represent endogenous BRCA1a (p110) and BRCA1b (p100) proteins

BRCA1 associates with RNA polymerase II holoenzyme complex (Scully *et al*., 1997a) and CBP (CREB-binding protein) is a component of the holoenzyme (Nakajima *et al*., 1997). Previously, we have found BRCA1a/p110 and BRCA1b/p100 to interact both *in vitro* and *in vivo* with the carboxy-terminal domain of transcription factor CBP (Cui *et al*., 1998b). Since the BRCT domain is sufficient for p53 mediated transactivation of the p21 promoter, we speculated whether the BRCT domain could interact directly with CBP which would bring the RNA polymerase II holoenzyme into play. Our results using GST-pull down assays demonstrate direct physical interaction between GST-CBP2 and *in vitro* translated BRCT domain of BRCA1 (Figure 4a). These data are consistent with the notion that BRCA1 functions as a transcriptional co-activator.

In summary, our results suggest BRCA1a and BRCA1b proteins function as co-activators of p53 tumor suppressor protein similar to BRCA1. This study demonstrates for the first time the presence of a second p53 interaction domain in the carboxy terminal BRCT domain of BRCA1 (Figure 4b), which is sufficient for activation of p53 dependent transactivation of the p21$^{WAF1/CIP1}$ promoter. Previously, based on its homology with p53 binding protein 53BP1, it was speculated that the BRCT domain could bind p53 (Koonin *et al*., 1996; Bork *et al*., 1997) and our result

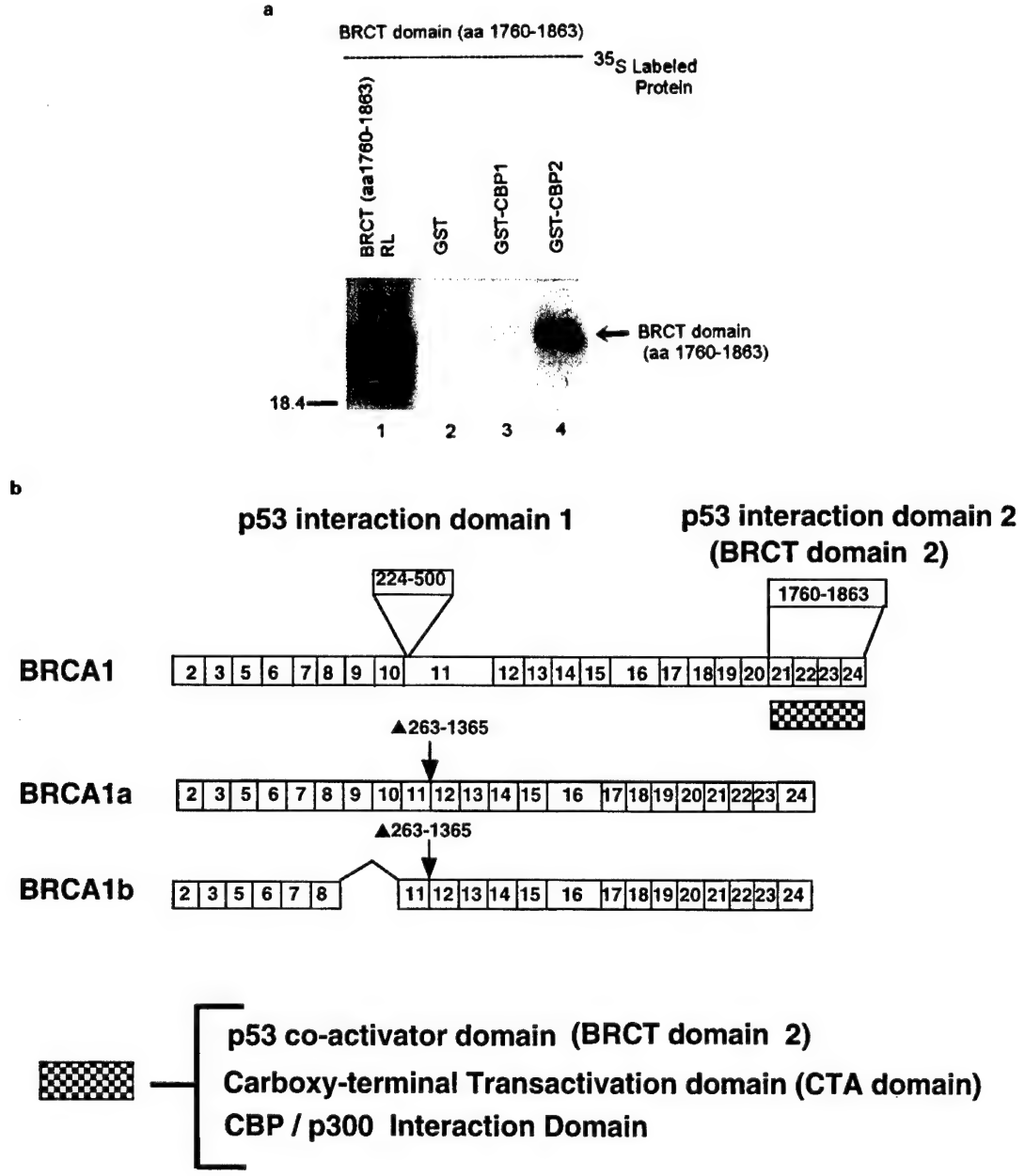

Figure 4 (**a**) BRCT domain (aa 1760–1863) interacts *in vitro* with CBP2 (aa 1620–1877). GST, GST-CBP1 or GST-CBP2 were incubated with *in vitro* translated ^{35}S-labeled BRCT domain (aa 1760–1863) and subjected to GST-pull down assay as described previously (Cui *et al*., 1998b). The faint band in lane 3 represents weak binding of BRCT domains to GST-CBP1. (**b**) Schematic representation of the p53 interaction domains of BRCA1/BRCA1a and BRCA1b proteins

confirms this hypothesis. Since BRCA1a, BRCA1b, and BRCT domain (residues 1760–1863) proteins associate with CBP/p300 co-activator (Cui *et al.*, 1998b and this paper) and the C-terminal truncation, Y1853X, fails to bind to RNA polymerase II holoenzyme (Scully *et al.*, 1997a) nor activate transcription (Somasundaram *et al.*, 1997; Chapman and Verma, 1996; Monteiro *et al.*, 1996; Ouchi *et al.*, 1998; Zhang *et al.*, 1998), we speculate that one of the mechanisms by which BRCA1 proteins function is through recruitment of CBP/p300 associated HAT/FAT activity for acetylation of p53 to specific promoters resulting in transcriptional activation. Our results demonstrate binding of the BRCT domain of BRCA1 (residues 1760–1863) to the central region of p53 (amino acids 79–307) but not to the C-terminal region (amino acids 319–393), which was earlier shown to bind to the N-terminal region of exon 11 of BRCA1 (amino acids 224–500). This central region of p53 contains the sequence specific DNA binding domain which is required for the tumor suppressor function of p53 protein (Ko and Prives, 1996 and references therein). The majority of p53 missense mutations in tumors are present within this central region. The binding of the BRCT domain of BRCA1 proteins to p53 suggests that it may be involved in p53 mediated tumor suppression; apoptosis, and DNA repair. It is possible that the BRCT domain may be involved in determining the specificity of p53 binding to high and low affinity p53 binding sites resulting in either p53-dependent transcriptional activation or repression. It is conceivable that the BRCT domain may activate p53 by inducing a conformational change resulting in increased DNA binding and transactivation. Our findings suggest that the BRCT domain binding to p53 may be mediated by CBP/p300, since it binds to the same region on BRCA1 and this complex may in turn be linked to RNA polymerase II holoenzyme complex. In fact, 87% of mutations that have been reported in familial breast cancer disrupt the BRCT domains by truncation of the protein (Couch and Weber, 1996), while some others disrupt the domains by missense mutations. It remains to be seen what effect these cancer-predisposing mutations have on the p53 binding and co-activator function of BRCA1 proteins. The p53 binding/co-activator property of BRCT domain can be used as an assay for detecting functionally relevant alterations in patients with BRCA1 mutations. It is possible that lack of binding of disrupted BRCA1 proteins to p53 in patients with mutations in the BRCT domain could lead to the development of breast cancer. The binding of the BRCT domain to p53 suggests that other BRCT domain containing proteins involved in cell cycle checkpoint functions responsive to DNA damage may similarly interact with p53. Lastly, although we have shown BRCA1a/1b to activate p53 dependent transcription of the p21 promoter in episome-based assays, the question whether the same holds true for endogenous p21 remains to be investigated.

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Structure and expression of variant BRCA2a lacking the transactivation domain

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Abstract. BRCA1 and BRCA2 are tumor suppressor genes shown to be involved in 90% of familial breast cancers and also known to be involved in ovarian and prostate cancers. Both BRCA1 and BRCA2 gene products are regulated in a cell cycle-dependent manner and have potential transactivation function. Here, we show that BRCA2 undergoes differential splicing giving rise to a novel variant protein BRCA2a, lacking putative transcriptional activation domain. Both BRCA2a and BRCA2 are expressed at high levels in thymus and testis but moderate levels in mammary gland and prostate suggesting that BRCA2a and BRCA2 may have a role in the development and differentiation of these tissues.

Introduction

Germ-line mutations in autosomal dominant susceptibility genes are responsible for up to 10% of all breast cancers (1,2). Mutations in breast cancer susceptibility genes, BRCA1 and BRCA2, could account for up to 90% of familial breast cancers (3-5). Recently BRCA1 and BRCA2 have also been shown to be associated with ovarian and prostate cancers. Interestingly, unlike BRCA1, BRCA2 is associated with male breast cancer (4). The BRCA2 gene is composed of 27 exons and encodes a protein of 3418 amino-acids with no significant homology to any known protein (6,7). Expression of both BRCA1 and BRCA2 was shown to be cell cycle regulated and expressed at high levels in late G1 and S-phase (8,9). Recently exon 3 of BRCA2 was shown to function as a transcriptional activation domain suggesting that BRCA2 may have a functional role in the regulation of transcription (10). Similar transcriptional activation function was also shown to be present in BRCA1 (11-13). Previously we have shown that BRCA1 is trans-ported into the nucleus in the absence of serum and interacts with transcriptional factor E2F, cyclins and cdks suggesting a role for BRCA1 in cell cycle regulation (14). BRCA2 and BRCA1 proteins were shown to interact with Rad 51 suggesting that they may play a role in DNA repair (15-18).

Recently we have shown that both BRCA1 and BRCA2 interact with CBP *in vivo* and *in vitro* suggesting that both these proteins may regulate transcription through CBP (19,20). Because of coordinated expression of BRCA1 and BRCA2 genes and association of these genes in the common breast cancer phenotype, it appears that BRCA1 and BRCA2 may function in a similar pathway. Recently, we have shown that BRCA1 induces apoptosis suggesting a novel function in the regulation of apoptosis of cells (21,22). It remains to be seen whether BRCA2 plays a similar role in apoptosis. Both BRCA1 and BRCA2 proteins may have multicellular functions such as transcriptional activation, DNA repair and regulation of apoptosis.

In this study, we have cloned alternatively spliced BRCA2 cDNA and characterized them by nucleotide sequence analysis. We demonstrate that this differentially spliced BRCA2a transcript has lost transcriptional activation domain as a result of alternative splicing giving rise to BRCA2a with potential dominant negative pathophysiology. Interestingly, BRCA1 was also shown to encode multiple products as a result of alternative splicing (13,14,21,23-25).

Materials and methods

Molecular cloning of BRCA2 and BRCA2a cDNAs. cDNAs were obtained by the reverse transcription of total RNA from BT-474 cells using cDNA kit (Takara). BRCA2 cDNAs were amplified by PCR using appropriate 5' and 3' primers and cloned into a pcDNA3 vector. These cDNAs were characterized by restriction mapping and nucleotide sequence analysis.

RNase protection assay. RNase protection assay was carried using Ribonuclease Protection assay kit (Ambion Inc., Austin TX) as described by the manufacturer. Briefly, the templates were subcloned, linearized and transcribed in 20 µl of *in vitro* transcription mixture containing 5 µl of α-^{32}P-rUTP to obtain radiolabelled probes. These radiolabelled RNA probes were purified by gel electrophoresis. Approximately $5x10^5$ cpm of the probe was mixed with 20 µg of human breast, prostate, testis and thymus RNA (Clontect, Palo Alto, CA) and the

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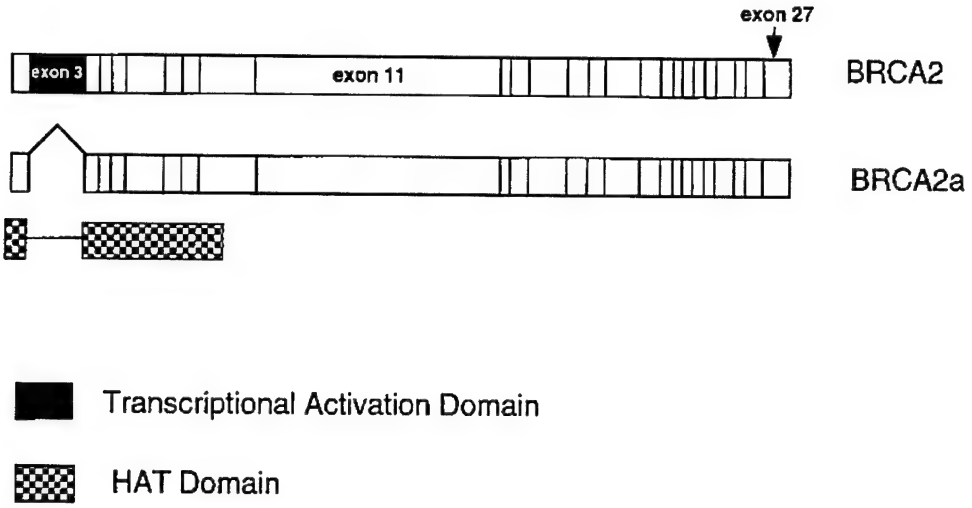

Figure 1. Deletion of BRCA2 transcriptional activation domain in BRCA2a as a result of alternative splicing. Figure is not drawn to scale.

volume of the reaction mixture was adjusted to 30 µl by 1X hybridization buffer. The hybridization mixture was heated to 95°C for 5 min and then incubated at 45°C overnight. RNase digestion was performed for 1 h at 37°C and the reaction was stopped by the addition of RNase inactivation buffer (kit). The protected fragments were extracted by centrifugation. The pellets were suspended in gel loading buffer, heated to 90°C for 3 min. The reaction products were analyzed by polyacrylamide gel electrophoresis using 5% polyacrylamide/8M urea gels. The gel was dried and subjected to autoradiography.

Results and Discussion

In order to understand the function of BRCA2, we have cloned several cDNAs by RT PCR and characterized these cDNAs by nucleotide sequence and restriction map analysis. Our results demonstrate that one of the cDNAs (BRCA2a) showed alternative splicing resulting in the deletion of exon 3 (Fig. 1). Previously this exon was shown to contain a potential transcriptional activation domain, which suggested that BRCA2 may function as a transcriptional factor (10). Similar potential for transcriptional activation was attributed to BRCA1 proteins (11-13). Since BRCA2a has lost transcriptional activation domain, it might compete with native BRCA2 in terms of DNA binding or interaction with other transcriptional factors resulting in dominant negative effect on transcription activation function of BRCA2. Such dominant negative variants are also seen in other transcriptional activators (26). Therefore, BRCA2a may represent a potential dominant negative variant which may regulate the putative transcriptional activation properties of BRCA2 proteins. Alternatively, BRCA2a may have other functions which do not need transactivation function.

We performed RNase protection analysis to study the expression of BRCA2 and BRCA2a in different types of tissues. For this, we have used the 459 nucleotide probe (Fig. 2a). The predicted 313 nucleotide fragment (corresponding to BRCA2a) and the 255 and 58 nucleotide fragments corresponding to BRCA2 were observed in thymus and testis (Fig. 2, lanes 3 and 4). However, moderate to low level of expression was observed in the case of mammary gland and prostate (Fig. 2, lanes 5 and 6). It appears both BRCA2 and BRCA2a are expressed at similar levels in the tissues tested suggesting both forms of BRCA2 may have a functional role in cell growth and differentiation of testis, mammary gland, prostate and thymus.

In summary, we have presented the results supporting that BRCA2 is alternatively spliced, giving rise to a variant BRCA2a protein which lacks transactivation domain. To our knowledge this is the first report demonstrating the presence of variant BRCA2 protein. Since BRCA2a variant lacks transcriptional activation domain, it can potentially interfere with transcriptional activation properties of BRCA2 by competing with BRCA2 for protein-protein interactions and/or DNA binding. Such variant proteins were also seen in the case of other transcriptional factors. It is possible that BRCA2a may regulate the functional properties of BRCA2. Therefore, it becomes important to study the patient DNA samples for mutations outside the coding region (introns, promoters etc.) as they may alter differential splicing pattern of BRCA2 leading to overexpression of BRCA2a. This overexpression of BRCA2a may interfere with normal BRCA2 function and result in cellular transformation. In support of this hypothesis, large deletions that disrupt exon 3 of BRCA2 were observed in patients of breast and ovarian cancers (27).

Our recent results have shown that both BRCA2 and BRCA2a have histone acetyltransferase activity (HAT) (20). These results suggest that domains responsible for HAT activity and the transcriptional activation function of BRCA2 do not overlap (Fig. 1). Since BRCA2 and BRCA2a are HAT proteins, it is possible both BRCA2 and BRCA2a function as transcriptional co-factors. BRCA1 was shown to function as a transcriptional co-factor of p53 (28,29). Recently, we have observed that BRCA2 binds to CBP and function as transcriptional co-factors of p53 (Siddique and Reddy, unpublished results). It is possible that some transcriptional

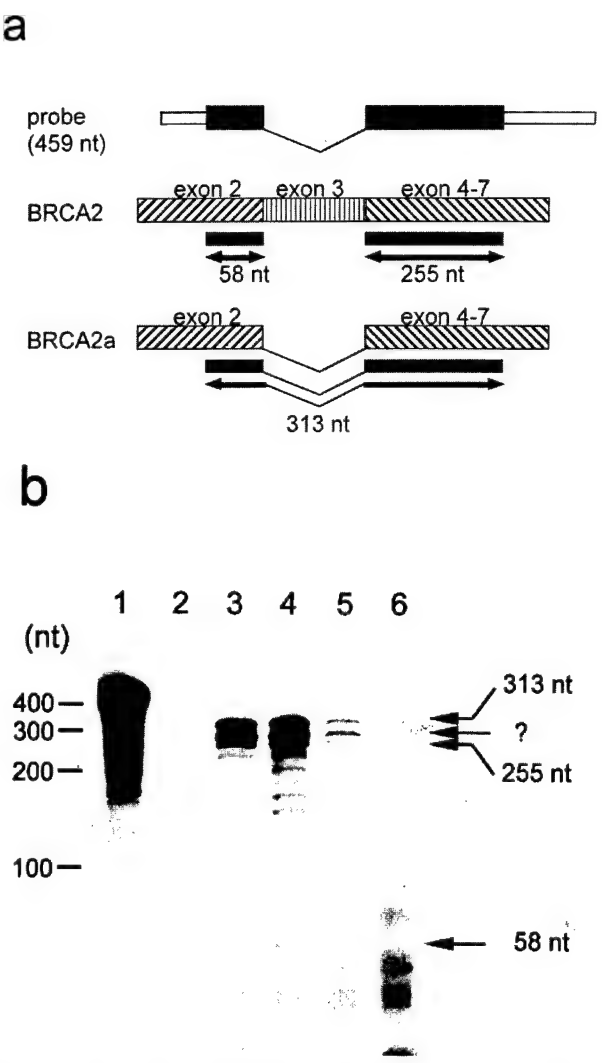

Figure 2. a) Schematic representation of antisense RNA probe used for RNase protection assay. The expected protected fragments from BRCA2 and BRCA2a transcripts are shown. b) Analysis of the expression of BRCA2a in different human tissues by RNase protection assay. This assay was carried out on 20 µg of total RNA using Ambion RPA kit. Lane 1, probe; Lane 2, tRNA; Lane 3, thymus, Lane 4, testis; Lane 5 mammary gland; Lane 6, prostate. Lanes 1-4 are the result of short exposure (1 day) and lanes 5 and 6 are of longer exposure (3 days). Protected 313 nucleotides (corresponding to the alternatively spliced BRCA2a) and 255 and 58 nucleotide fragments (corresponding to the BRCA2) are shown by arrows.

activators may use BRCA2 and BRCA2a as a transcriptional co-factors and utilize their HAT activity for the activation of gene expression. It is also possible that other transcriptional activators may use BRCA2 but not BRCA2a as a transcriptional co-factor because of the absence of the transcriptional activation domain in BRCA2a. Recent studies revealed that BRCA2 interacts with DNA repair protein Rad 51 (15-17). Earlier, we suggested that BRCA2-Rad 51 complex may disrupt nucleosomal structure using HAT activity of BRCA2 and thereby recognize damaged DNA prior to DNA repair process (20). For such a mechanism, there may not be a need for transactivation function. Therefore, BRCA2 and BRCA2a may have distinct functions. Rad 51-BRCA2a (which lacks transactivation domain) complex may play a role in DNA repair whereas BRCA2 may play a role in the regulation of transcription. It remains to be seen whether both isoforms of BRCA2 play a role in this DNA repair phenomenon. Alternatively, BRCA2 proteins may participate in DNA repair and tumor suppression through BRCA2 target genes.

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c-Fos oncogene regulator Elk-1 interacts with BRCA1 splice variants BRCA1a/1b and enhances BRCA1a/1b-mediated growth suppression in breast cancer cells

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Elk-1, a c-Fos protooncogene regulator, which belongs to the ETS-domain family of transcriptional factors, plays an important role in the induction of immediate early gene expression in response to a variety of extracellular signals. In this study, we demonstrate for the first time the *in vitro* and *in vivo* interaction of Elk-1 with BRCA1 splice variants BRCA1a and BRCA1b using GST-pull down assays, co-imunoprecipitations/Western blot analysis of cell extracts from breast cancer cells and mammalian two-hybrid assays. We have localized the BRCA1 interaction domain of Elk-1 protein to the conserved ETS domain, a motif involved in DNA binding and protein–protein interactions. We also observed binding of BRCA1 proteins to other ETS-domain transcription factors SAP1, ETS-1, ERG-2 and Fli-1 but not to Elk-1 splice variant ΔElk-1 and c-Fos protooncogene. Both BRCA1a and BRCA1b splice variants function as growth suppressors of human breast cancer cells. Interestingly, our studies reveal that although both Elk-1 and SAP-1 are highly homologous members of a subfamily of ETS domain proteins called ternary complex factors, it is only Elk-1 but not SAP-1 that can augment the growth suppressive function of BRCA1a/1b proteins in breast cancer cells. Thus Elk-1 could be a potential downstream target of BRCA1 in its growth control pathway. Furthermore, we have observed inhibition of c-Fos promoter activity in BRCA1a transfected stable breast cancer cells and over expression of BRCA1a/1b attenuates MEK-induced SRE activation *in vivo*. These results demonstrate for the first time a link between the growth suppressive function of BRCA1a/1b proteins and signal transduction pathway involving Elk-1 protein. All these results taken together suggest that one of the mechanisms by which BRCA1a/1b proteins function as growth/tumor suppressors is through inhibition of the expression of Elk-1 target genes like c-Fos. *Oncogene* (2001) **20,** 1357–1367.

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Introduction

BRCA1, a familial breast and ovarian cancer susceptibility gene is mutated in approximately 45% of the families with inherited breast cancers and 80–90% of families with inherited breast and ovarian cancers (Couch and Weber, 1996; Easton *et al.*, 1995; Ford *et al.*, 1995; Miki *et al.*, 1994; Struewing *et al.*, 1997). Recently, loss of heterozygosity (33%) at the BRCA1 locus and reduced expression of BRCA1 mRNA was also observed in sporadic breast cancers (Katsama *et al.*, 2000; Ozcelik *et al.*, 1998). The BRCA1 gene spans approximately 100 kb on chromosome 17q21.3, is composed of 22 coding exons and encodes a protein of 1863 amino acids (Miki *et al.*, 1994). We have recently isolated and characterized two new naturally occurring variants of BRCA1, BRCA1a/p110 and BRCA1b/p100 (Wang *et al.*, 1997) which are abundantly expressed in both non-malignant and tumor-derived breast cells (Lu *et al.*, 1996), Rao *et al.*, unpublished results. Other groups have independently identified these isoforms and have designated BRCA1a as BRCA1-Δ11b (Wilson *et al.*, 1997), BRCA1s (Lu *et al.*, 1996) and BRCA1b as BRCA1s-9, 10 (Lu *et al.*, 1996). Wild-type BRCA1 and splice variants BRCA1a/1b proteins bind to a number of cellular proteins. The unique amino-terminal RING finger domain interacts with BARD1 (Wu *et al.*, 1996), BAP1 (Jensen *et al.*, 1998), BIP (Wang *et al.*, 1997), E2F transcriptional factors, cyclins and cyclin-dependent kinases (Wang *et al.*, 1997). Two 'BRCT' (BRCA1 carboxyl terminus) repeats present at the carboxy-terminal region of BRCA1 proteins (Bork *et al.*, 1997; Callebaut and Mornon, 1997; Koonin *et al.*, 1996) are involved in transcription activation, growth inhibition and tumor suppression (Chapman and Verma, 1996; Humphrey *et al.*, 1997; Monteiro *et al.*, 1996; Rao *et al.*, 1996; Thompson *et al.*, 1995). The BRCT domains of BRCA1 are targets for cancer associated mutations (Couch and Weber, 1996). Recently, we and others have shown the N- and C-terminal regions of BRCA1,

BRCA1a and BRCA1b proteins to activate transcription when fused to GAL4 DNA binding domain (Chapman and Verma, 1996; Cui *et al*., 1998b; Monteiro *et al*., 1996) and associate with RNA polymerase II holoenzyme (Scully *et al*., 1997a) and CBP/P-300 co-activator (Cui *et al*., 1998a), suggesting a role for BRCT domains in the regulation of transcription. The BRCT domain also binds to RNA helicase A (Anderson *et al*., 1998), ctBP interacting protein (Yu *et al*., 1998), tumor suppressor p53 protein (Chai *et al*., 1999; Somasundaram *et al*., 1997; Zhang *et al*., 1998) and BRCA2 protein (Chen *et al*., 1998). BRCA1 has also been shown to interact with DNA repair protein Rad51 (Scully *et al*., 1997b) and c-*myc* oncoprotein (Wang *et al*., 1998). Thus, the association with these proteins mediate the involvement of BRCA1 proteins in growth/tumor suppression (Holt *et al*., 1996; Humphrey *et al*., 1997; Rao *et al*., 1996; Thompson *et al*., 1995), induction of apoptosis (Shao *et al*., 1996), cell cycle control, transcriptional transactivation (Chai *et al*., 1999; Chapman and Verma, 1996; Monteiro *et al*., 1996; Zhang *et al*., 1998), DNA repair and the maintenance of genomic stability (Brugarolas and Jacks, 1997; Lane *et al*., 1995; Marquis *et al*., 1995; Scully *et al*., 1997b).

The Elk-1 gene is a member of a subfamily of ETS domain proteins called ternary complex factors (TCFs), i.e. Elk-1, SAP-1 and NET/ERP/SAP2/Elk-3 (Dalton and Treisman, 1992; Giovane *et al*., 1994; Hipskind *et al*., 1991; Lopez *et al*., 1994; Nozaki *et al*., 1996; Price *et al*., 1995; Rao *et al*., 1989) that associate with the serum response factor (SRF) to regulate the transcription of c-Fos protooncogene (Dalton and Treisman, 1992; Hipskind *et al*., 1991). The Elk-1 gene codes for two splice variants Elk-1 (Rao *et al*., 1989) and ΔElk-1 (Rao and Reddy, 1993) which function as sequence specific transcriptional activators (Bhattacharya *et al*., 1993; Rao and Reddy, 1992), are substrates for MAP kinases (Hill *et al*., 1993; Marais *et al*., 1993; Rao and Reddy, 1993) and JNK protein kinases (Gupta *et al*., 1996; Whitmarsh *et al*., 1995), and induce apoptosis (Shao *et al*., 1998). Elk-1 is an efficient substrate for all three classes of MAPK, unlike SAP-1 which is activated only by ERK and p38 MAPK, and Net is activated by expression of oncogenic Raf (Wasylyk *et al*., 1998). Despite the high degree of sequence similarity within the ETS domain (~80%) both Elk-1 and SAP1 proteins show different DNA binding specificities (Shore and Sharrocks, 1995). In this study, we have found ternary complex factor Elk-1 to interact with BRCA1 splice variants BRCA1a and BRCA1b proteins. We demonstrate *in vitro* and *in vivo* interactions between Elk-1 and BRCA1 proteins by using GST pull-down assays, co-immunoprecipitations/Western blot analysis and mammalian two-hybrid assays. BRCA1 proteins bind to the ETS DNA-binding domain of Elk-1. Interestingly, although both Elk-1 and SAP-1 bind to BRCA1 proteins, it is only Elk-1 but not SAP-1 that can enhance the growth suppressive function of BRCA1a/1b proteins in breast cancer cells. Furthermore, overexpression of BRCA1a and BRCA1b inhibits MEK-induced c-Fos SRE promoter activation *in vivo*. These findings suggest that BRCA1 proteins may function as growth/tumor suppressors by regulating the expression of c-Fos.

Results and Discussion

BRCA1 inhibits c-Fos promoter activity in breast cancer cells

One of the specific changes brought about by numerous oncogenes is the constitutive elevation of c-Fos proto-oncogene expression which may represent a causal event in the transformation process. Similarly, the c-Fos basal promoter was shown to be suppressed by wild type p53 tumor suppressor. We therefore determined the activity of the c-Fos SRE (a DNA promoter element whose activity is regulated by a variety of growth promoting events) by using a SRE-TK-CAT reporter in NIH3T3 or MCF-7 cells that have been transfected with either antisense RNA to BRCA1a (Rao *et al*., 1996) or BRCA1a (Shao *et al*., 1996). We observed high levels of CAT activity in BRCA1a antisense transfected NIH3T3 cells (Figure 1a). Similarly transfection of SRE-TK-CAT reporter plasmid in BRCA1a transfected MCF-7 cells showed ~98% reduction in the levels of CAT activity compared to parental pcDNA3 transfected MCF-7 cells, unlike PSV2CAT transfected cells (data not given). These results suggested that this effect may be unique to SRE-dependent reporters (Figure 1b). In fact, SRE activity is dependent upon the activation by phosphorylation of ternary complex factor Elk-1. The results also suggest that BRCA1 could directly or indirectly regulate the function of Elk-1 target genes like c-Fos, or Elk-1 could be a potential downstream target of BRCA1 proteins in the apoptotic or tumor suppressive pathway. These observations lead us to investigate whether Elk-1 associates with BRCA1 proteins.

BRCA1 interacts with Elk-1 and SAP-1 but not ΔElk-1 and c-Fos in vitro

To investigate whether BRCA1a/1b proteins associate directly with Elk-1, *in vitro* binding was performed with *in vitro* translated Elk-1 and GST-BRCA1a or GST-BRCA1b proteins using a GST fusion protein pull-down assay as described previously (Cui *et al*., 1998a; Wang *et al*., 1997). The *in vitro* translated ^{35}S-methionine-labeled Elk-1 protein was passed through GST-BRCA1a, GST-BRCA1b, and a GST control column. Elk-1 was found to bind specifically to GST-BRCA1a and GST-BRCA1b proteins but not to GST (Figure 2a). Similarly, we also studied the *in vitro* binding of another Elk-1 related protein SAP1a, ΔElk-1 and c-Fos to BRCA1a/1b proteins. *In vitro* translated full-length SAP1a (Figure 2b) unlike ΔElk-1 and c-Fos proteins bind to BRCA1a/1b proteins (Figure 2c and d). All these results suggest *in vitro* association of

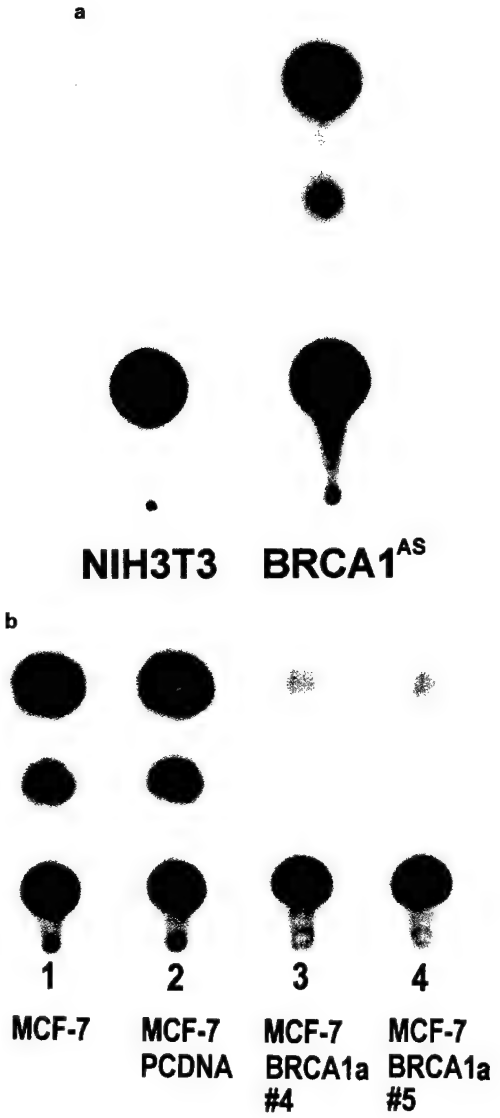

Figure 1 Functional assay for SRE activity in stable NIH3T3 and NIH3T3 BRCA1^{AS} cells (**a**) and in stable MCF-7 pcDNA and MCF-7 BRCA1a transfectants (**b**). pBLSRE-TK CAT reporter plasmid and pCH110 internal reference plasmids were co-transfected into the indicated cells using the Promega transfection kit as described (Rao and Reddy, 1993). The chromatogram represents a typical experiment

BRCA1 proteins with Elk-1 and SAP-1. We have further characterized the Elk-1 BRCA1a interaction *in vitro* by co-immunoprecipitation of *in vitro* synthesized Elk-1 and BRCA1a proteins *in vitro*. Full-length Elk-1 and BRCA1a proteins were translated together *in vitro* and subjected to immunoprecipitation using either anti-BRCA1 antibody or anti-Elk-1 antibody. BRCA1a protein was precipitated in the presence of Elk-1 (Figure 3a, lane 4). Similarly, Elk-1 protein was precipitated in the presence of BRCA1a (Figure 3a, lane 5) unlike IgG control (Figure 3a, lane 6). All these results suggest a specific interaction between the two proteins. To further confirm the association of BRCA1 proteins with endogenous Elk-1 protein *in vivo*, we incubated cell lysates obtained from a human breast cancer cell line CAL-51 with GST-immobilized on GSH beads and BRCA1a fusion protein conjugated GSH beads. The beads were washed and the bound proteins were subjected to Western blot analysis using Elk-1 specific polyclonal antibody as described previously (Shao *et al*., 1998). An ~62 kD band corresponding to the size of Elk-1 protein was detected in the lysates of GST-BRCA1a beads but not control GST beads (Figure 3b). These results suggest the association of BRCA1 proteins with endogenous Elk-1.

Localization of the BRCA1 binding domain of Elk-1

To map the region of Elk-1 which binds to BRCA1a/1b proteins, a series of carboxy- and amino-terminal Elk-1 deletion mutants expressed and purified as GST fusion proteins (Rao and Reddy, 1992) were incubated with *in vitro* translated full-length BRCA1b protein and subjected to GST pull-down assay. Our results suggested the binding of BRCA1b protein to the aminoterminal ETS domain (amino acids 1–89) of Elk-1 protein (Figure 4a). This winged-helix–turn-helix (wHTH) ETS domain of Elk-1, which is highly conserved among all ETS proteins, mediates both DNA binding (Rao and Reddy, 1992) and protein–protein interactions with itself (Drewett *et al*., 2000) or other proteins (Yates *et al*., 1999). If this ETS domain is sufficient for interaction with BRCA1 proteins, then one should expect all the other Ets related proteins to bind to BRCA1. We, therefore, studied the binding of GST-BRCA1a/1b proteins to *in vitro* translated full-length ETS-1, ERG-2, and Fli-1 using a GST pull-down assay. Our results suggest weak binding of ETS-1, ERG-2 and Fli-1 to BRCA1 proteins (Figure 4b, c,d).

BRCA1 Elk-1 interaction in vivo *in mammalian cells*

To investigate whether Elk-1 and BRCA1 could associate in mammalian cells, we performed a co-immunoprecipitation followed by Western blot analysis in co-transfected COS-7 cells. We co-transfected COS-7 cells with GAL4 Elk-1 (residues 1–205) and FLAG-epitope tagged full-length BRCA1a. Forty-eight hours post transfection, cell lysates were prepared and subjected to immunoprecipitation with FLAG antibody. This immuno-precipitated complex was subjected to SDS–PAGE followed by Western blotting with GAL4 (DBD) antibody. As shown in Figure 5a, Elk-1 was co-precipitated with the full-length FLAG BRCA1a (lane 3) using a FLAG antibody but not using an IgG antibody (lane 1). Taken together these results suggest that Elk-1 and BRCA1 proteins associate *in vivo*. To further document the *in vivo* binding of Elk-1 and BRCA1 proteins, we next showed that endogenous Elk-1 associates with endogenous BRCA1 proteins in CAL51 cells. Cell lysates from CAL51 cells were immunoprecipitated with Elk-1 antibody and the immunocomplexes were subjected to

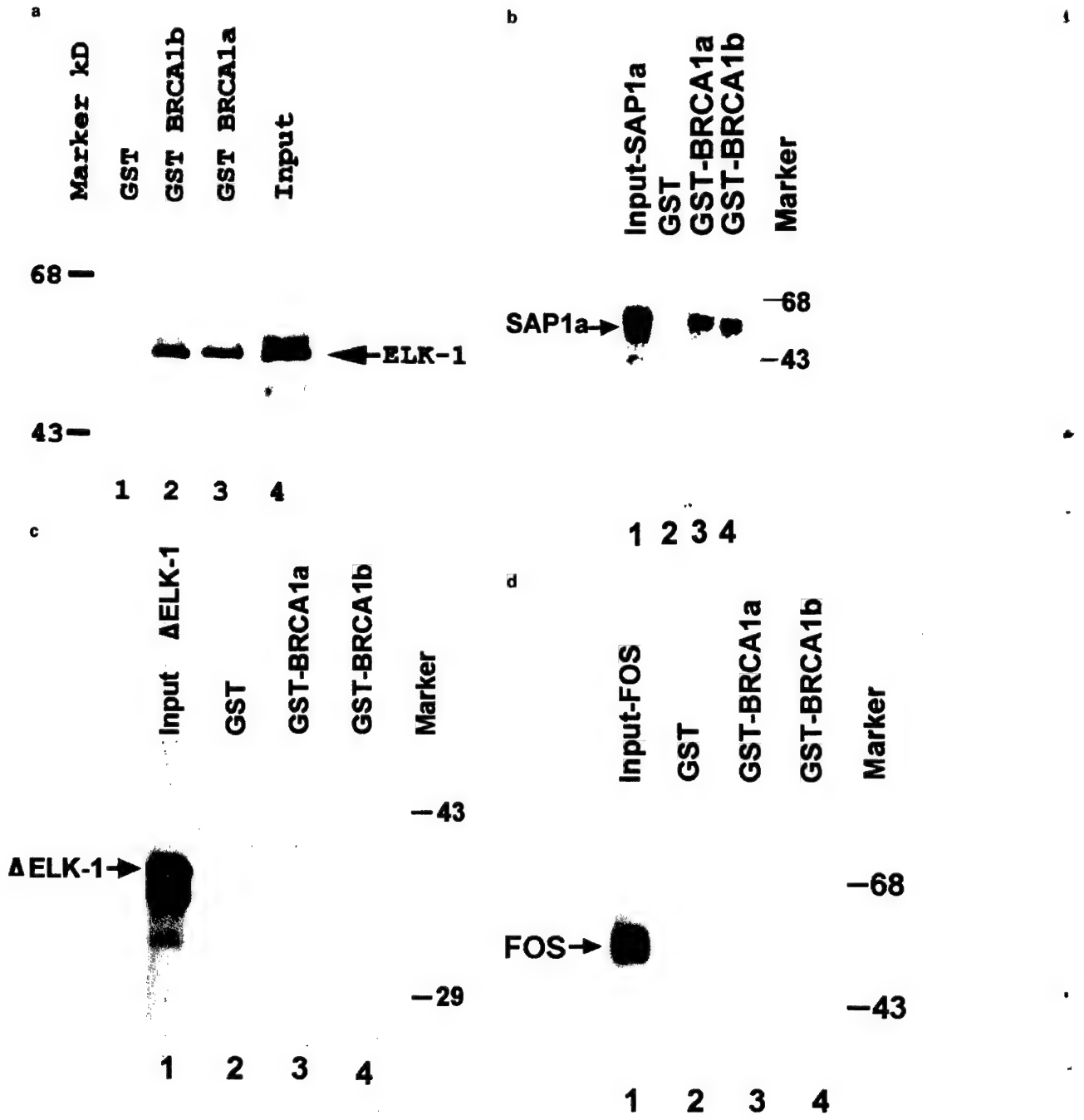

Figure 2 Direct physical association of Elk-1 and SAP1a but not ΔElk-1 and c-Fos with BRCA1a/1b proteins *in vitro*. GST, GST-BRCA1a, GST-BRCA1b proteins were expressed, purified on beads and incubated with *in vitro* translated ^{35}S-methionine-labeled full-length Elk-1 (**a**), or full-length SAP1a (**b**), or ΔElk-1 (**c**), or c-Fos (**d**), and subjected to GST pull-down assay as described (Chai *et al*., 1999). In lane 4 (**a**), lane 1 (**b**), lane 1 (**c**), lane 1 (**d**) 1/12th of the *in vitro* translated protein that was used for binding was loaded directly

Western blot analysis using BRCA1 antibodies. As shown in Figure 5b, lane 2, both BRCA1a and BRCA1b proteins were co-precipitated with the Elk-1 antibody but not with a control IgG antibody (Figure 5b, lane 1). Having established that the ETS domain of Elk-1 can directly associate with full-length BRCA1a and BRCA1b proteins *in vitro*, we next studied the interaction *in vivo* using the mammalian two-hybrid system. We have subcloned the full-length BRCA1a and BRCA1b cDNA's in frame with the GAL-4 DNA binding domain vector. The ETS domain of Elk-1 has been cloned in frame with the VP16 activation domain VP16-Elk-1 (1–89). COS-7 cells were co-transfected with VP16-Elk-1 and GAL4 BRCA1a or GAL4 BRCA1b and pG5E1bCAT as a reporter. Figure 5c shows that using the two-hybrid assay, the VP16- Elk-1

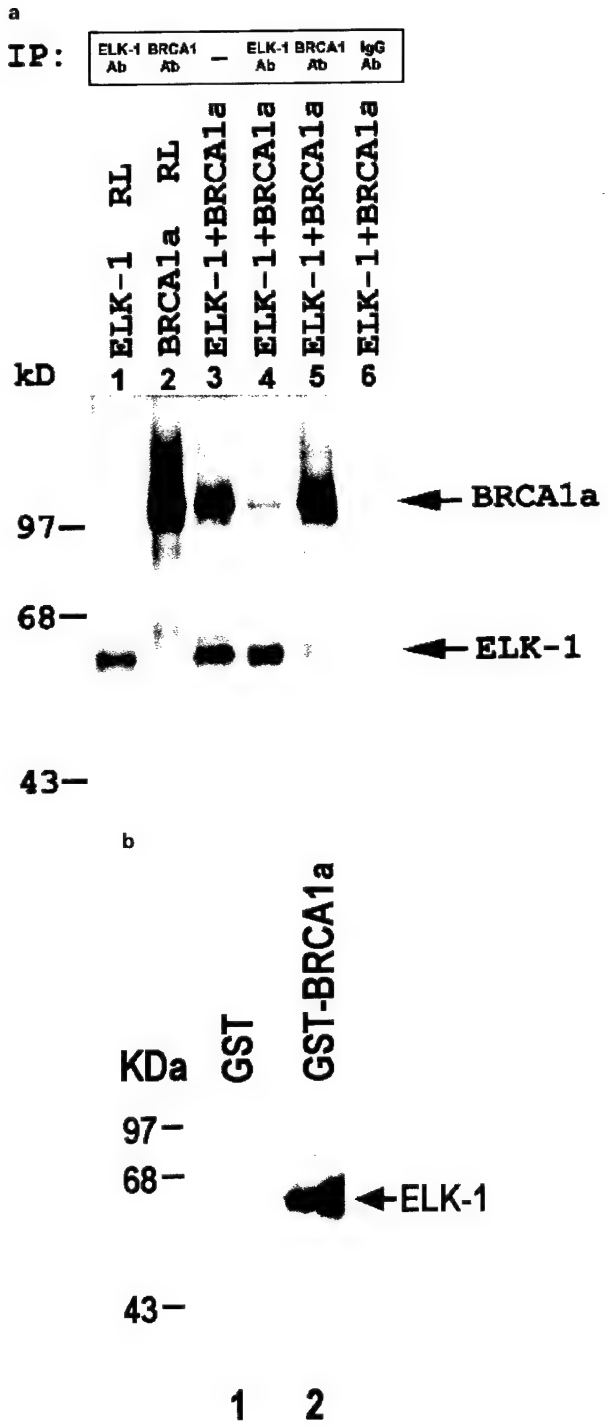

Figure 3 Elk-1 interacts with BRCA1a protein *in vitro*. (**a**) Co-immunoprecipitation of BRCA1a and Elk-1 *in vitro*. *In vitro* translated ^{35}S-methionine-labeled full-length Elk-1 and BRCA1a proteins were mixed and immunoprecipitated using Elk-1, BRCA1 polyclonal antibodies, IgG control and subjected to SDS polyacrylamide gel analysis. Lane 3 loaded directly without immunoprecipitation. (**b**) Association of BRCA1a protein with endogenous Elk-1. Unlabeled cell extracts from CAL51 cells were incubated with GST conjugated GSH beads (lane 1) or with GST conjugated BRCA1a beads (lane 2). The beads were washed and subjected to 10% SDS–PAGE and Western blot analysis using antibodies specific to Elk-1 as described (Wang *et al*., 1997)

protein stimulates GAL4 BRCA1a and GAL4 BRCA1b activity consistent with the *in vitro* binding results (Figure 3a). As expected from our previous work (Cui *et al*., 1998a), this activation is not seen with the pSGVP16 vector alone or with both full-length GAL4 BRCA1a and GAL BRCA1b (Figure 5c). These results further confirm the *in vivo* interactions of BRCA1 and Elk-1 proteins.

Elk-1 but not ΔElk-1 and SAP1a enhances the growth suppressive activity of BRCA1a/1b in human breast cancer cells

We and others have shown the BRCA1, BRCA1a and BRCA1b proteins to function as growth/tumor suppressors and induce apoptosis of human breast, ovarian and prostate cancer cells (Fan *et al*., 1998; Holt *et al*., 1996; Rao *et al*., 1996; Shao *et al*., 1996; Zhang *et al*., 1998; and Rao, unpublished results). We therefore studied whether Elk-1 may change BRCA1a/b mediated growth suppression of human breast cancer cells. We co-transfected BRCA1a or BRCA1b and Elk-1 cDNA's into MCF-7 breast cancer cells and analysed their effect on G418 colony formation. We have used MCF-7 cells lines because this cell line lacks one copy of a 2 Mb region which contains the BRCA1 gene, the growth of these cells is inhibited by the over-expression of BRCA1 (Holt *et al*., 1996), BRCA1a/1b proteins (Figure 6, Rao, unpublished results), this cell line contains wild type p53 and expresses Elk-1 protein. The expression of BRCA1a alone (pcDNA BRCA1a), BRCA1b alone (pcDNA BRCA1b) or Elk-1 alone (pcDNA Elk-1) resulted in reduction in the number of G418 colonies formed when compared to pcDNA3 vector (Figure 6), unlike ΔElk-1 (pcDNA ΔElk-1) or SAP1a alone (pcDNA SAP1a). The co-expression of BRCA1a/1b and Elk-1 (Figure 6a,b) but not ΔElk-1 (Figure 6a,b) or SAP1a (Figure 6c) significantly decreased the number of colonies. These results suggest that Elk-1 augments the growth suppressive function of BRCA1a/1b proteins in human breast cancer cells and demonstrates for the first time that BRCA1a/1b proteins like BRCA1 can function as growth suppressors of human breast cancer cells. Similar enhancement in growth suppression of BRCA1 was previously observed for another protein BAP1 (Jensen *et al*., 1998). Alternately, it could be possible that the results that we have obtained using Elk-1 and BRCA1 may represent an additive effect rather than an interactive effect. Future studies should resolve these issues. Thus, the majority of exon 11 sequences that are deleted in BRCA1a/1b isoforms (residues 263–1365) are not necessary for the growth suppression function of BRCA1 proteins. Our results also show that altering Elk-1 activity by elevated levels of expression of Elk-1 in human breast cancer cells can result in growth suppression of human breast cancer cells. These studies also reveal how highly homologous ETS proteins like Elk-1 and SAP1a can exhibit distinct biological properties.

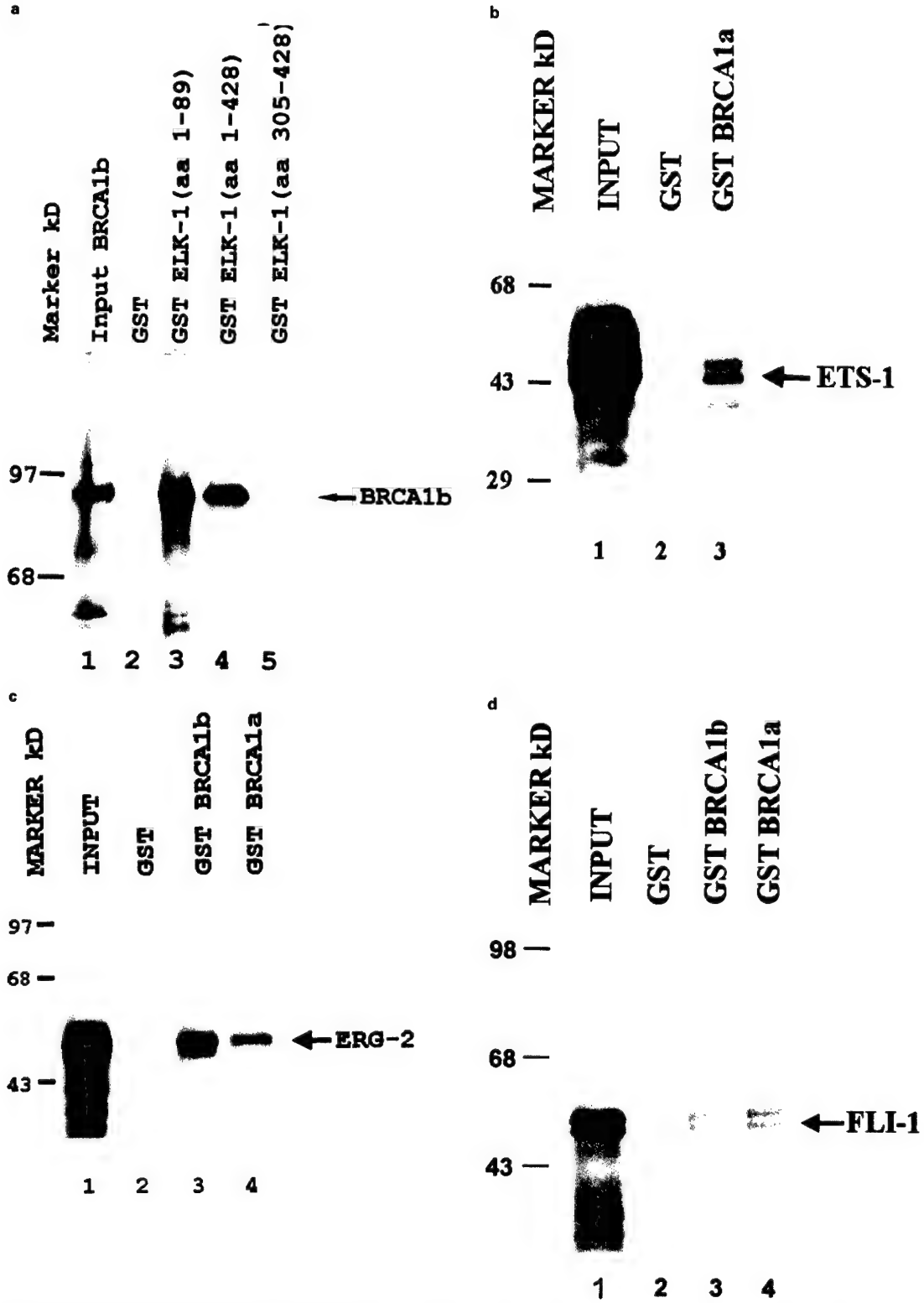

Figure 4 BRCA1a/1b proteins bind to the ETS domain of Elk-1, ETS-1, ERG-2 and Fli-1 proteins. (**a**) GST, GST-Elk-1 (amino acids 1–428), GST Elk-1 (amino acids 1–89) and GST Elk-1 (amino acids 305–428) proteins were expressed, purified on beads and incubated with *in vitro* translated ^{35}S-methionone labeled BRCA1b and subjected to GST pull-down assay as described (Wang *et al.*, 1997). GST, GST-BRCA1a, GST-BRCA1b proteins were purified on beads and incubated with *in vitro* translated ^{35}S-methionine labeled ETS-1 (**b**) or ERG-2 (**c**) or Fli-1 (**d**) and subjected to GST pull-down assay as described (Wang *et al.*, 1997). In lane 1 (**a**), lane 1 (**b**), lane 1 (**c**) and lane 1 (**d**), 1/10th of the *in vitro* protein used for binding was loaded directly

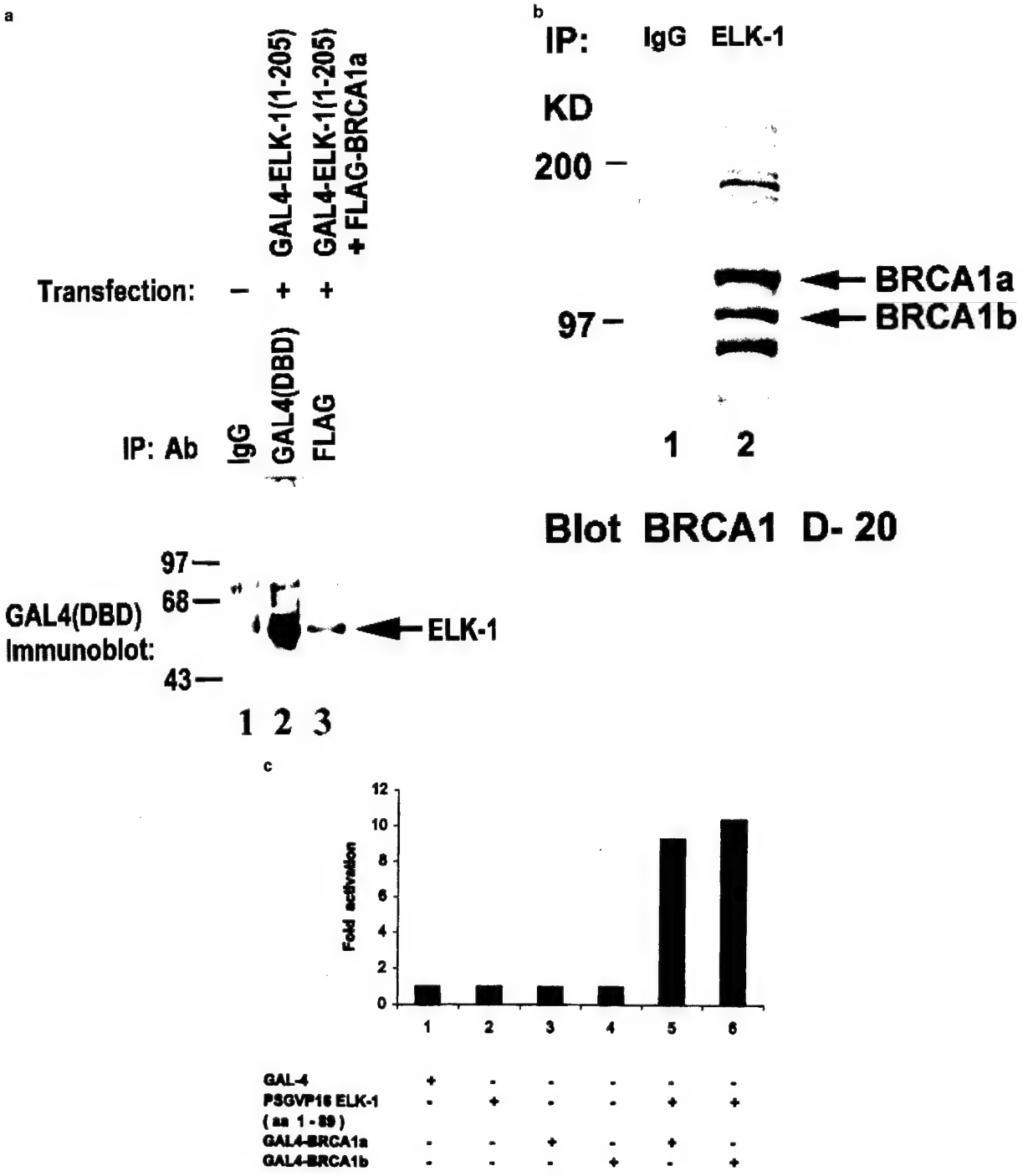

Figure 5 *In vivo* interaction of Elk-1 with BRCA1 proteins. (**a**) COS-7 cells were co-transfected with GAL4-Elk-1 and FLAG epitope tagged BRCA1a plasmids. After 48 h of transfection, cell lysates were subjected to immunoprecipitation using an IgG antibody (lane 1) or GAL4 (DBD) antibody (lane 2) or an anti-FLAG antibody (lane 3). The immunocomplex was then separated on an SDS–PAGE and subjected to Western blot analysis using an anti-GAL4 (DBD) antibody. Elk-1 was co-precipitated with FLAG-BRCA1a by the anti-FLAG antibody. (**b**) CAL-51 cells were used to study the endogenous interaction between BRCA1a/1b and Elk-1. CAL-51 cell lysates were subjected to imunoprecipitation using an IgG antibody (lane 1) or Elk-1 antibody (lane 2). The immunocomplex was then separated on an 8% SDS–PAGE and subjected to Western blot analysis using BRCA1 (D-20) antibody. BRCA1a/1b proteins are indicated by arrows. (**c**) Full-length BRCA1a and BRCA1b interact with the ETS domain of Elk-1 in a mammalian two-hybrid system. COS-7 cells were co-transfected with 6 μg of 17 MzCAT reporter plasmid 1 μg of VP16 expression plasmids and 1 μg of GAL4 expression plasmids as described (Cui *et al*., 1998a). The CAT activity shown represents fold activation compared with the VP16 vector alone

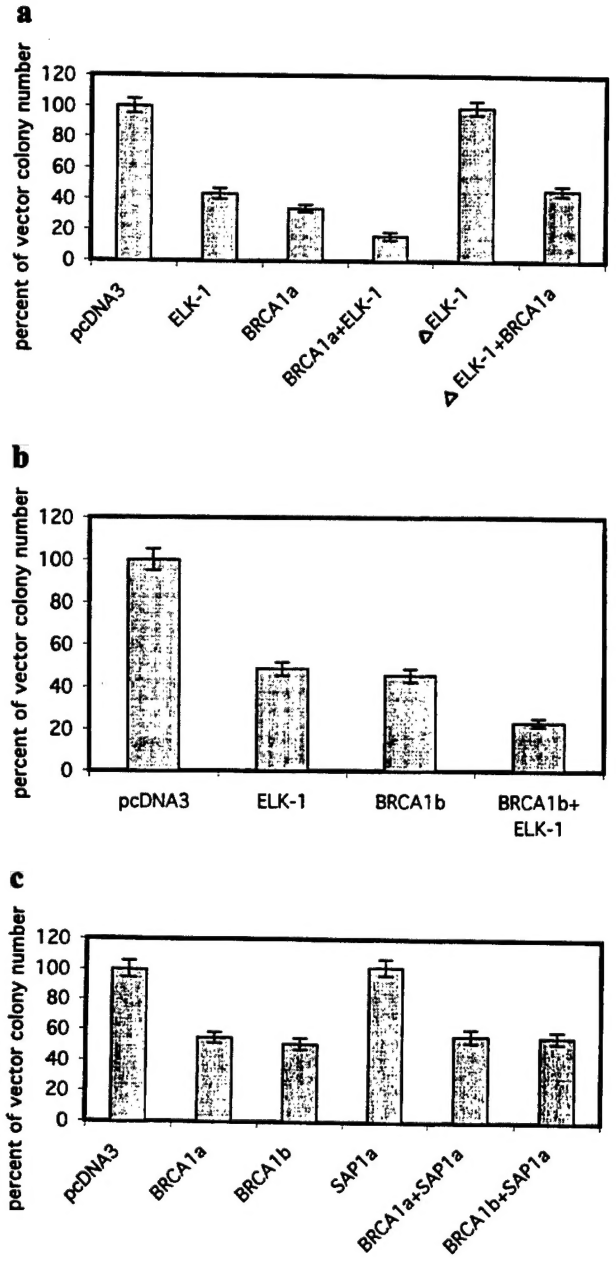

Figure 6 Elk-1 unlike ΔElk-1 and SAP1a enhances BRCA1a/1b mediated growth suppression of MCF-7 cells. MCF-7 (1×10^6) were co-transfected with (**a**) and (**b**) pcDNA3 or pcDNA3 Elk-1 or pcDNA3 ΔElk-1 or (**c**) pcDNA3 SAP1a either alone or in the presence of pcDNA3 BRCA1a or pcDNA3 BRCA1b plasmid constructs as shown. Twenty-one days later, cells were stained with crystal violet and colonies counted. The experiment was repeated four times using different preparations of plasmid DNA. Inhibition of colony formation is shown in percentage with the number of neomycin-resistant colony resulting from pcDNA3 transfection set as 100%

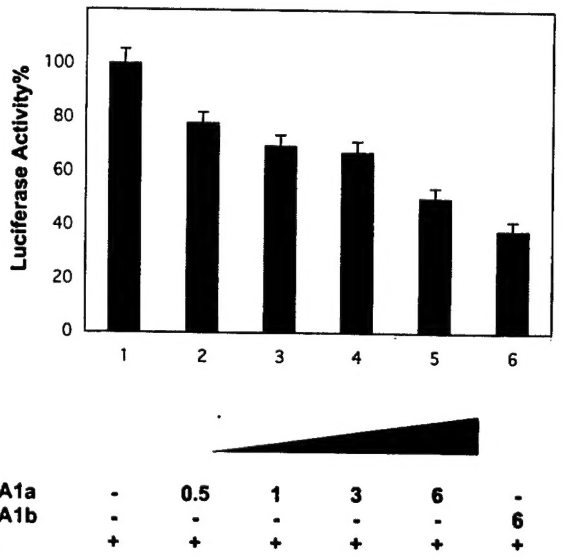

Figure 7 BRCA1a and BRCA1b proteins inhibit ERK-mediated induction of the c-Fos SRE reporter. NIH3T3 cells were co-transfected with SREx2-luc reporter plasmid. pCMVMEK-1 either in the absence or presence of pcDNA BRCA1a/1b. All values and standard errors were calculated from averages of triplicate samples and are representative of three independent experiments

BRCA1 inhibits ERK-1 mediated induction of the c-Fos promoter via the SRE

Elk-1 plays a key role in c-Fos induction following stimulation with growth factors and mitogens. Our results suggest that BRCA1 proteins inhibit c-Fos promoter activity in breast cancer cells that have been stably transfected with BRCA1a. We therefore studied the effect of over expression of BRCA1a/1b proteins on the activity of an SRE-driven reporter in NIH3T3 cells. A constitutively active MEK protein was co-transfected to stimulate the c-Fos reporter via the extracellular signal-related kinase (ERK) pathway. Co-transfection of increasing amounts of BRCA1a/1b caused a dose-dependent reduction in the activity of the reporter in response to MEK induction (Figure 7). These results demonstrate that the BRCA1 proteins may function by inhibiting MEK-inducible c-Fos promoter activation. We can speculate that this inhibition can occur either via the Elk-1 SRE-SRF ternary complex on the c-Fos promoter, or via competing for a common factor(s).

Physiological relevance of Elk-1-BRCA1 interaction to c-Fos activity

Full-length Elk-1 binds to BRCA1a/1b proteins *in vitro* and *in vivo* and enhances the growth suppression properties of BRCA1a/1b proteins in colony formation assays. These results demonstrate for the first time a key link between the growth suppressive function of BRCA1a/1b proteins and signal transduction pathway involving Elk-1 proteins. Indeed, over expression of BRCA1a/1b proteins in human breast cancer cells and NIH3T3 cells causes inhibition of the activity of the c-Fos promoter, indicating that Elk-1 could be a target for BRCA1a/1b mediated inhibition of c-Fos promoter activity. Currently, the mechanism

of tumor suppression by BRCA1 is not well understood. Previous studies have suggested that one mechanism by which BRCA1 protein may contribute to cell cycle arrest and growth/tumor suppression is through the induction of $p21^{WAF1/CIP1}$ (Chai *et al.*, 1999; Somasundaram *et al.*, 1997). It may be possible that BRCA1 functions are either regulated or mediated through interactions with cellular proteins like Elk-1, and disruption of this protein–protein association by germ-line mutations in BRCA1 could be one important step in tumorigenesis. Alternately, wild type BRCA1 could cooperate with Elk-1 in growth inhibition unlike the mutant form. Future work will be directed towards studying whether Elk-1/BRCA1 interaction can be disrupted by BRCA1 mutations that segregate with breast cancer and studying the mechanism of down regulation of c-Fos transcriptional activity mediated by BRCA1. This would provide substantial evidence for the physiological relevance of this interaction in human breast/ovarian and prostate cancers.

Materials and methods

GST pull-down assay

GST-BRCA1a and GST-BRCA1b were expressed and purified from *E-coli* as reported previously (Wang *et al.*, 1997). For *in vitro* binding assay ^{35}S-methionine labeled *in vitro* translated full-length Elk-1 (Rao *et al.*, 1989), SAP1a (Dalton and Treisman, 1992), ΔElk-1 (Rao and Reddy, 1993), c-Fos (Abate *et al.*, 1991), ETS-1 (Reddy and Rao, 1990), ERG-2 (Rao *et al.*, 1987) and Fli-1 (Rao *et al.*, 1993) proteins were diluted in TNN buffer (50 mM Tris-Hcl-pH7.4, 150 mM Nacl, 0.5% IGEPAL CA-630) containing 1 mm PMSF, 10 μg/ml leupeptin, 3% aprotinin and 1 mM sodium orthovanadate. The solutions were precleared with GSH beads for 1 h and then incubated with GST or GST-BRCA1a or GST-BRCA1b beads for 2 h at 4°C. The beads were then washed six times in TNN buffer and heated in SDS sample buffer and loaded on a 10% SDS–PAGE. Similarly, GST-Elk-1 (residues 1–428), GST-Elk-1 (residues 1–89) and GST-Elk-1 (residues 305–428) were expressed and purified as described previously (Rao and Reddy, 1992). For *in vitro* binding experiments, full-length *in vitro* translated BRCA1b was tested for binding to the different deletion mutants of Elk-1 as described above for GST pull-down assay. The gels were fixed, treated with enhance, dried and exposed to X-ray films or exposed and scanned using a Fuji BioImaging analyser.

Co-immunoprecipation and Western blot analysis

COS-7 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS). These cells were transfected with GAL4-Elk-1 (residues 1–205) or GAL4-Elk-1 (residues 1–205) and FLAG-BRCA1a to a total of 20 μg DNA using the calcium phosphate mammalian transfection system (Invitrogen). Forty-eight hours after transfection, cells were lysed in lysis buffer and the supernatant was subjected to immunoprecipitation with either normal IgG or GAL4 (DBD) antibody or FLAG antibody as described previously (Wang *et al.*, 1997). SDS–PAGE and Western blotting using GAL4 (DBD) polyclonal antibody (Santa Cruz Biotechnology) was carried out subsequently as described previously (Cui *et al.*, 1998a). Similarly, CAL-51 cells were used to study the endogenous interaction of Elk-1 with BRCA1 proteins. CAL-51 cells lysates were immunoprecipitated with either normal IgG or polyclonal anti-Elk-1 antibody (New England Biolabs) and the complexes were run on 8% SDS–PAGE gel and subjected to Western blot analysis using D-20 polyclonal anti BRCA1 antibody (Santa Cruz Biotechnology).

In vitro *co-immunoprecipitation*

In vitro translation of full-length Elk-1 (pcDNA-Elk-1) and full-length BRCA1a (pcDNA-BRCA1a) was done with the TNT quick-coupled transcription/translation system (Promega). *In vitro* co-immunoprecipitation was performed by mixing 40 μl of rabbit reticulocyte lysate containing radiolabelled full-length Elk-1 with 40 μl of reticulocyte lysate containing radiolabeled full-length BRCA1a or with 40 μl of an uncharged reticulocyte lysate. Equal aliquots of samples were diluted in buffer and immunoprecipitated at 4°C for 2 h with 1 μl of either Elk-1 antibody (#9182, NEB) or BRCA1 polyclonal antibody (SC-641, Santa Cruz) or normal IgG. Protein A Sepharose beads were added and the incubation was continued for 16 h. The beads were then pelleted by brief centrifugation and washed three times in RIPA buffer, boiled for 2 min in SDS loading buffer and pelleted by centrifugation. The supernatant was then loaded on a SDS 10% polyacrylamide gel.

Cell culture, transfection and reporter gene assays

NIH3T3, NIH3T3-$BRCA1^{AS}$ cells, MCF-7, MCF-7-pcDNA, MCF-7-BRCA1a #4, MCF-7-BRCA1a #5, and COS-7 cells were maintained in DMEM supplemented with 10% FBS and 200 μg/ml G418 as required. For reporter gene assays, c-Fos SRE-driven reporter (SRE-TK CAT 6 μg) were co-transfected with CH110 (5 μg) using the calcium phosphate method (Promega) as described previously (Rao and Reddy, 1993). DNA concentrations were normalized with Gem-3 vector. Similarly NIH3T3 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). Triplicate transfections were carried out in 100 mm petri dishes using SRE × 2-Luc reporter (5 μg) vector and pCMVMEK-1 either in the absence or presence of 0.5, 1.0, 3.0, 6.0 μg of pcDNA BRCA1a/1b, respectively. Luciferase assays were carried out as described by the manufacturer (Promega). All values and standard errors were calculated from averages of triplicate samples and are representative of three independent experiments. For two-hybrid analysis in mammalian cells, full-length BRCA1a and BRCA1b cDNAs were subcloned in frame with the GAL4 DNA binding domain vector obtained from P Chambon (Cui *et al.*, 1998a). ETS domain of Elk-1 (residues 1–89) was subcloned in frame with the VP16 transactivation domain vector pSGVP16 (Rao and Reddy, 1993). G5E1bCAT reporter plasmid was a gift from T Kouzarides. COS-7 cells were co-transected with pSGVP16 Elk-1 alone (1 μg) or in the presence of GAL4-BRCA1a or GAL4-BRCA1b and G5E1bCAT reporter plasmid (6 μg) as described (Cui *et al.*, 1998a). After 48 h post transfection, cell extracts were prepared and subjected to β-galactosidase and CAT assays as described (Cui *et al.*, 1998a). The CAT assays were quantitated with a Fuji PhosphoImager. The experiments were repeated several times.

Colony suppression assay

MCF-7 cells, at 1.5×10^5 cells/10 cm dish were transfected with 20 μg of plasmid DNAs (pcDNA3, pcDNA-BRCA1a/1b, pcDNA-Elk-1, pcDNAΔElk-1, pcDNA-SAP1a, etc.) using the calcium phosphate mammalian transfection system from Promega. The next day, cells were washed twice in 1 × PBS and re-fed with fresh medium (DMEM supplemented with 10% FBS). Twenty-four hours later, the cells were trypsinized and plated into six 10 cm petri dishes containing complete medium and 600 μg/ml G418. Cells were fed with medium containing G418 every 3–4 days. Cells were fixed approximately 18–21 days after transfection in methanol-acetic acid (3:1) for 1 h at −20°C and stained for colonies with crystal violet.

Preparation of total cell extract, protein binding and Western blot analysis

CAL-51 cells were washed in ice-cold PBS and scraped into 1 ml of TNN buffer (Wang *et al.*, 1997) and lysed by rotating for 30 min. The lysates were centrifuged at 14 000 *g* for 30 min and subjected to GST pull-down assay. For protein binding assay, cell extracts were precleared overnight with GSH beads and then incubated with either GSH protein conjugated GSH beads or GST-BRCA1a. Proteins were incubated with GSH beads for 2 h at 4°C. The beads were washed in TNN buffer and boiled in SDS sample buffer and loaded on a 10% PAGE. After electro transfer onto PVDF membrane, it was probed with antibodies specific to Elk-1 as described previously (Wang *et al.*, 1997).

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